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# THE JOURNAL OF HYGIENE

EDITED BY

GEORGE H. F. NUTTALL, M.D., PH.D., SC.D., ~~F.R.S.~~

QUICK PROFESSOR OF BIOLOGY IN THE UNIVERSITY OF CAMBRIDGE

IN CONJUNCTION WITH

JOHN S. HALDANE, M.D., LL.D., F.R.S.

LATE READER IN PHYSIOLOGY IN THE UNIVERSITY OF OXFORD

CHARLES J. MARTIN, M.B., D.Sc., F.R.S.

DIRECTOR OF THE LISTER INSTITUTE, LONDON

J. C. G. LEDINGHAM, M.B., D.Sc.

BACTERIOLOGIST-IN-CHIEF, LISTER INSTITUTE, LONDON

G. S. GRAHAM-SMITH, M.D.

UNIVERSITY LECTURER IN HYGIENE, CAMBRIDGE

MAJOR GREENWOOD, M.R.C.P. (LOND.)

MEDICAL OFFICER (MEDICAL STATISTICS), MINISTRY OF HEALTH

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SOME FACTORS INFLUENCING THE ACTIONS OF DYES  
AND ALLIED COMPOUNDS ON BACTERIA<sup>1</sup>.

BY G. S. GRAHAM-SMITH, M.D.

*(From the Medical School, University of Cambridge.)**(With Plate I and six charts.)*

THE experiments recorded in this paper were undertaken in order to ascertain the effects of certain dyes and allied organic compounds on three selected species of bacteria, *Staphylococcus aureus*, *B. coli* and *B. pyocyaneus*. These experiments show that in cultures the results are influenced by many factors, and appear to suggest that the varying results of the use of these compounds in the treatment of wounds may be ascribed to some extent to the different conditions prevailing in each case.

The chief organic substances examined were diaminotrimethylacridinium chloride, termed for the sake of brevity, Homoflavine, Quinone and Crystal Violet. Homoflavine is more easily prepared on a technical scale than the ordinary acriflavine, with which it is homologous, being made from the acridine produced from metatoluylenediamine by combination with a methyl ester; both it and Crystal Violet have been produced on a manufacturing scale by Messrs Levinstein, Ltd, of Manchester, and pure material provided by this firm has been used in the present investigation. The other organic compounds named in the table on p. 4 have been carefully purified by Professor W. J. Pope; none of the dye stuffs comprised in this list were double compounds with metallic salts and their compositions are stated in Green's *Organic Colouring Matters*.

During the last few years many investigations on the bactericidal effects of dyes have been undertaken. Browning, Gulbransen, Kennaway and Thornton (1917), Dakin and Dunham (1917), and Nicholls (1917), employed 0·7 % peptone, Fleming (1917), Hewlett (1917), Morgan (1918), Taylor (1917) and Wright (1917) used "broth," Drummond and McNee (1917) "glucose broth," Morgan (1918) serum broth, Churchman (1912, 1913), Churchman and Michael (1912), Krumwiede and Pratt (1914) and Teague (1918) "ordinary agar" and Browning and Gilmour (1914) "peptone water agar." Presumably all these media contained the usual quantity of peptone. The use of peptone<sup>2</sup>

<sup>1</sup> A Report to the Medical Research Committee, April 25, 1918.

<sup>2</sup> Browning, Gulbransen and Thornton (1917) have shown that "for some unascertained reason the bacterial potency of flavine for staphylococcus in dilute peptone water shows considerable variations in an extended series of experiments."



has been avoided in these experiments, and consequently the results of these workers are not strictly comparable with those quoted in this paper.

Browning, Gulbransen, Kennaway and Thornton (1917), Fleming (1917) and Morgan (1918) used ox serum, Dakin and Dunham (1917) horse serum, and horse serum together with muscle extract, and Hewlett (1917) human serum. Blood was employed by Dakin and Dunham (1917), Fleming (1917) and Morgan (1918), pus by Fleming (1917) and Hewlett (1917), and milk by Hewlett (1917). None of these fluids were employed in the present experiments.

Varying results in preliminary experiments made it evident that to obtain comparable results media of simple and constant composition would be required, and that an arbitrary time limit would have to be adopted. As a fluid medium neutral meat extract and as a solid neutral meat extract agar were chosen and the actions of the compounds on the three species of organisms growing in the former and on the surface of the latter compared.

The meat extract was prepared from bullock's heart muscle after removing the fat, fascia, etc. To each 100 grms. of minced meat 250 c.c. of water were added, the fluid slowly boiled for one hour, filtered through filter paper and sterilised in the autoclave. The clear extract so obtained requires 0.08 c.c. of N/10 soda to render it neutral to neutral red.

On this medium all the organisms mentioned grow very well.

Tubes containing 1 c.c. of meat extract, 0.08 c.c. of N/10 soda, 3.5 c.c. of distilled water were prepared and sterilised by boiling. When cool 0.5 c.c. of a solution of the compound in distilled water and lastly a drop of an emulsion in sterile distilled water of the organism from a 24 hour old agar culture grown at 37° C. were added, and the culture incubated at 37° C. Care was taken to make emulsions as uniform in numbers of bacteria as possible, and on many occasions the organisms present in the cultures at the beginning of the experiment were estimated by means of plate cultures. The numbers in most of the experiments varied between 4,000 and 2,000 organisms per drop. The naked eye results were noted after 24 and 48 hours' incubation and to confirm them subcultures were sown on agar plates with a standard loop (0.01 c.c.).

In order to compare the results when the organisms were grown on the surface of agar 1 c.c. of meat extract, 2 c.c. of melted agar (2 % in distilled water), 0.08 c.c. of N/10 soda and 1.5 c.c. of distilled water were placed in tubes and sterilised by boiling. When cooled to 60° C. 0.5 c.c. of a solution of the compound was added. After thorough mixing the contents of the tube were poured into Petri dishes<sup>1</sup> and allowed to set. With the aid of a platinum loop three streaks of strong emulsions of the three organisms in distilled water

<sup>1</sup> Small Petri dishes divided according to the method devised by Churchman (1912) were employed in order to reduce the quantity of medium used and to facilitate comparison. Instead of metal divisions cardboard strips, cemented to the bottoms and sides of the dishes with water agar, were utilised.

were made across the surface of the medium. The cultures were incubated at 37° C. and the results were noted after 24 and 48 hours' incubation.

Table I (p. 4) gives the results of these experiments after 48 hours' incubation, a + indicating visible growth in the fluid medium or growth on the agar though only evidenced by the presence of a single colony, and a 0 the failure of the organism to grow.

In the agar series it frequently happened that though no growth was visible after 24 hours' incubation a few or even numerous colonies were found on examining the plate after 48 hours' incubation. Consequently the results obtained after 24 hours' incubation differ considerably from those obtained after 48 hours' incubation (Plate I, figs. 1-20). Since *in the agar series* further incubation seldom produces any change, the time limit of 48 hours was chosen, and unless otherwise stated the results recorded indicate the findings under these conditions.

*In the fluid medium* good growth may occur on subsequent days in tubes which show no evidence of growth in 2 days.

Table I, in which the results are tabulated in the order of the action of the compounds on staphylococci growing on the surface of neutral agar, shows that (1) under these conditions the effects of the compounds are not strictly correlated to their chemical relationship, (2) the substances most toxic to staphylococci have little toxicity to *B. coli* or *B. pyocyaneus*, and (3) of the three organisms *B. pyocyaneus* is the most resistant.

The second part of the table gives the action of the compounds on the organisms growing in meat extract, and shows that (1) under these conditions different values are obtained for many of the compounds, (2) a different order in efficiency is found, and (3) some of the compounds exhibit marked toxicity to *B. coli* and to a lesser degree to *B. pyocyaneus*.

The differences between the tables are especially noteworthy since agar has no nutritive value and the quantity of nutrient material (meat extract) is the same in both series.

Since Crystal Violet exhibits the greatest toxicity towards staphylococci, Quinone the greatest toxicity towards *B. coli* and *B. pyocyaneus*, when growing on agar, and homoflavine the greatest toxicity towards these organisms in meat extract, these three compounds were selected for further investigation.

### Homoflavine.

From Table I it will be seen that on agar the toxicity of homoflavine towards staphylococci is somewhat greater than that of methylhomoflavine or methylhomoacridine, while the toxicity of these three compounds towards *B. coli* and *B. pyocyaneus* is similar. In meat extract the toxicity of all three towards staphylococci is similar, but homoflavine and methylhomoflavine are more toxic to *B. coli* and to *B. pyocyaneus* than methylhomoacridine.

Table I.

Showing the least concentrations of the following compounds which cause inhibition of growth for 48 hours at 37° C.

	Neutral agar			Neutral meat extract		
	Staphylo- cocci	<i>B. coli</i>	<i>B. pyro- cyanus</i>	Staphylo- cocci	<i>B. coli</i>	<i>B. pyro- cyanus</i>
1. Crystal violet	1: 3,250,000	1: 10,000	1: 10,000	1: 10,000,000	—	—
2. Brilliant red Rhoduline	1: 1,000,000	1: 1,000	1: 1,000	1: 500,000	1: 10,000	1: 1,000
3. Irisamine	1: 900,000	1: 1,000	1: 1,000	1: 1,100,000	1: 1,000	1: 1,000
4. Metaphenylene Blue B	1: 200,000	1: 1,000	1: 1,000	1: 900,000	1: 10,000	1: 10,000
5. Pyronine G	1: 150,000	1: 1,000	1: 1,000	1: 200,000	1: 20,000	1: 10,000
6. Pararosaniline hydro- chloride	1: 70,000	1: 1,000	1: 1,000	1: 60,000	1: 1,000	1: 1,000
7. Quinone	1: 70,000	1: 95,000	1: 30,000	1: 60,000	1: 100,000	1: 10,000
8. Hydroquinone	1: 60,000	1: 40,000	1: 10,000	1: 20,000	1: 20,000	1: 10,000
9. Safranin S	1: 50,000	1: 1,000	1: 1,000	1: 70,000	1: 20,000	1: 1,000
10. Acridine red	1: 50,000	1: 10,000	1: 1,000	1: 30,000	1: 1,000	1: 1,000
11. Isonitrophenol	1: 40,000	1: 20,000	1: 10,000	1: 20,000	1: 20,000	1: 10,000
12. Chrysoidine T	1: 20,000	1: 10,000	1: 1,000	1: 30,000	1: 10,000	1: 1,000
13. Homoflavine	1: 14,000	1: 9,000	1: 1,000	1: 300,000	1: 250,000	1: 30,000
14. Methylhomoflavine	1: 10,000	1: 10,000	1: 1,000	1: 300,000	1: 250,000	1: 30,000
15. Methylhomoacridine	1: 10,000	1: 10,000	1: 1,000	1: 300,000	1: 200,000	1: 10,000
16. Toluidine blue acid	1: 10,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
17. Congo red acid	1: 10,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
18. Phosphine R	1: 10,000	1: 1,000	1: 1,000	—	—	—
19. Rhodamine B	1: 10,000	1: 1,000	1: 1,000	1: 10,000	1: 1,000	1: 1,000
20. Rhodamine S	1: 10,000	1: 1,000	1: 1,000	1: 70,000	1: 1,000	1: 1,000
21. Erythrosine	1: 10,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
22. Alizarin red	1: 1,000	1: 1,000	1: 1,000	1: 10,000	1: 1,000	1: 1,000
23. Orange G	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
24. Night blue basic	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
25. Diamine blue 3 B	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
26. Crystal scarlet 6 R	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
27. Benzö blue 6 B neutral	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
28. Diaminogeno blue G	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
29. Quinoline yellow S	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
30. Acid violet 6 B	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
31. Magenta S	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
32. Thioflavine S	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
33. Tartrazine	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
34. Trypan red	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
35. Trypan blue	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
36. Auramine O	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
37. Patent blue	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000
38. Naphthol green	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000	1: 1,000

Concentrations greater than 1:1,000 were not tested, and the sign 1:1,000 indicates that good growth took place at a concentration of 1:1,000.

It will be noticed that the influence on staphylococci of Nos. 2 and 8 is much greater on agar than in meat extract, of numbers 10, 11, 16, 17, and 21 slightly greater, while the influence of numbers 1, 4, 13, 14, 15, and 20 is much greater in meat extract than on agar, and of numbers 3, 5, 9, 12, and 22 slightly greater. The influence of numbers 6, 7, and 19, and probably of numbers 23-38, is similar in both media.

*The effects of varying the proportion of nutrient material and the reaction of the medium.*

In order to ascertain the effects of varying the proportion of the nutrient material and the reaction of the medium several series of experiments were carried out both in meat extract and on meat extract agar. In each case the total quantity of the medium employed was 5 c.c., but the quantity of nutrient material (meat extract) varied between 0.5 c.c. and 2 c.c. For example at neutrality to neutral red the meat extract series was made up in the following manner.

	Meat extract	N/10 soda	Distilled water	Solution of homoflavine
A	0.5*	0.04	3.96	0.5
B	1.0	0.08	3.42	0.5
C	1.5	0.12	2.88	0.5
D	2.0	0.16	2.34	0.5

\* In this and other tables describing the composition of media the figures indicate the quantities of ingredients used in c.c.

And the agar series as follows.

	Meat extract	Agar (2%)	N/10 soda	Distilled water	Solution of homoflavine
A	0.5	2.0	0.04	1.96	0.5
B	1.0	2.0	0.08	1.42	0.5
C	1.5	2.0	0.12	0.88	0.5
D	2.0	2.0	0.16	0.34	0.5

In order to vary the reaction on the alkaline side additional quantities of N/10 soda (0.1, 0.2, 0.3, 0.4, 0.5 c.c.) were added beyond the neutral point with a corresponding diminution in the amount of water. On the acid side either no addition was made, or in some cases N/10 hydrochloric acid (0.05, 0.1 and 0.15 c.c.) was added with a corresponding diminution in the amount of water.

Thus in the experiments with the fluid medium containing 1 c.c. of meat extract the whole series was made up in the following manner.

	Meat extract	N/10 soda	N/10 HCl	Distilled water	Solution of homoflavine
I	1.0	—	0.1	3.4	0.5
II	1.0	—	0.05	3.45	0.5
III	1.0	—	—	3.5	0.5
IV	1.0	0.08	—	3.42	0.5
V	1.0	0.18	—	3.32	0.5
VI	1.0	0.28	—	3.22	0.5
VII	1.0	0.38	—	3.12	0.5
VIII	1.0	0.48	—	3.02	0.5
IX	1.0	0.58	—	2.92	0.5

The concentration of homoflavine causing complete inhibition of growth during 48 hours' incubation at 37°C. is given in Table II and Charts I and II.

## Action of Dyes on Bacteria

Table II.

Showing the concentration of homoflavine required to cause complete inhibition of growth after 48 hours' incubation at 37° C. on agar and in meat extract of different reactions and containing different quantities of nutrient material.

Quantity of acid or alkali added	Quantity of meat extract	Agar			Meat extract		
		<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
0.1 N/10 HCl	1.0	1: 1,500	1: 4,000	1: 500	—	1: 200,000	1: 30,000
0.05 "	1.0	1: 1,500	1: 8,000	1: 500	1: 150,000	1: 250,000	1: 20,000
No addition	0.5	1: 9,000	1: 18,000	1: 500	1: 300,000	1: 500,000	1: 40,000
	1.0	1: 4,500	1: 10,000	1: 500	1: 150,000	1: 300,000	1: 20,000
	1.5	1: 3,500	1: 9,000	1: 500	1: 100,000	1: 200,000	1: 17,000
	2.0	1: 2,000	1: 6,000	1: 500	1: 100,000	1: 150,000	1: 13,000
0.08 N/10 soda	0.5	1: 11,000	1: 20,000	—			
per c.c. of meat extract*	1.0	1: 9,000	1: 14,000	—			
	1.5	1: 8,000	1: 11,000	—			
	2.0	1: 7,000	1: 10,000	—			
	0.5	1: 35,000	1: 30,000	—			
0.18 N/10 soda	1.0	1: 12,000	1: 17,000	—			
	1.5	1: 8,000	1: 15,000	—			
	2.0	1: 7,000	1: 10,000	—			
	0.5	1: 60,000	1: 75,000	—			
0.28 "	1.0	1: 20,000	1: 20,000	—			
	1.5	1: 9,000	1: 15,000	—			
	2.0	1: 8,000	1: 12,000	—			
	0.5	1: 100,000	1: 160,000	1: 1,500	1: 1,800,000	1: 1,800,000	1: 110,000
0.38 "	1.0	1: 45,000	1: 40,000	1: 1,500	1: 1,600,000	1: 1,400,000	1: 70,000
	1.5	1: 11,000	1: 16,000	1: 1,500	1: 1,100,000	1: 1,100,000	1: 50,000
	2.0	1: 10,000	1: 15,000	1: 1,500	1: 1,000,000	1: 800,000	1: 40,000
	0.5	1: 290,000	1: 300,000	—			
0.48 "	1.0	1: 90,000	1: 90,000	—			
	1.5	1: 15,000	1: 21,000	—			
	2.0	1: 14,000	1: 21,000	—			
	0.5	1: 300,000	1: 320,000	1: 1,500	1: 2,000,000	1: 2,000,000	1: 130,000
0.58 "	1.0	1: 145,000	1: 160,000	1: 1,500	1: 1,800,000	1: 1,800,000	1: 110,000
	1.5	1: 18,000	1: 23,000	1: 1,500	1: 1,400,000	1: 1,600,000	1: 100,000
	2.0	1: 17,000	1: 26,000	1: 1,500	1: 1,200,000	1: 1,400,000	1: 90,000

\* 1 c.c. of meat extract requires 0.08 c.c. N/10 soda to neutralise it to neutral red. In cultures containing 0.5, 1.5 and 2.0 c.c. of meat extract proportionate quantities of N/10 soda were added. In the alkaline series 0.1, 0.2, 0.3, 0.4, 0.5 c.c. of N/10 soda were added beyond the quantities sufficient to bring the reaction to neutrality. For the sake of lucidity these figures have been omitted in Table II and Charts I, II and III, only the quantities added to cultures containing 1 c.c. being quoted.

It will be seen from Table II and Charts I and II that under all conditions the smaller the amount of nutrient material present the greater the efficiency of the homoflavine solution, and that in regard to *B. coli* and staphylococci its efficiency is greatly increased by the addition of small quantities of N/10 soda. On agar with the larger quantities of nutrient material the successive increments of soda have less effect than with the smaller quantities. In meat extract cultures this phenomenon is much less marked.

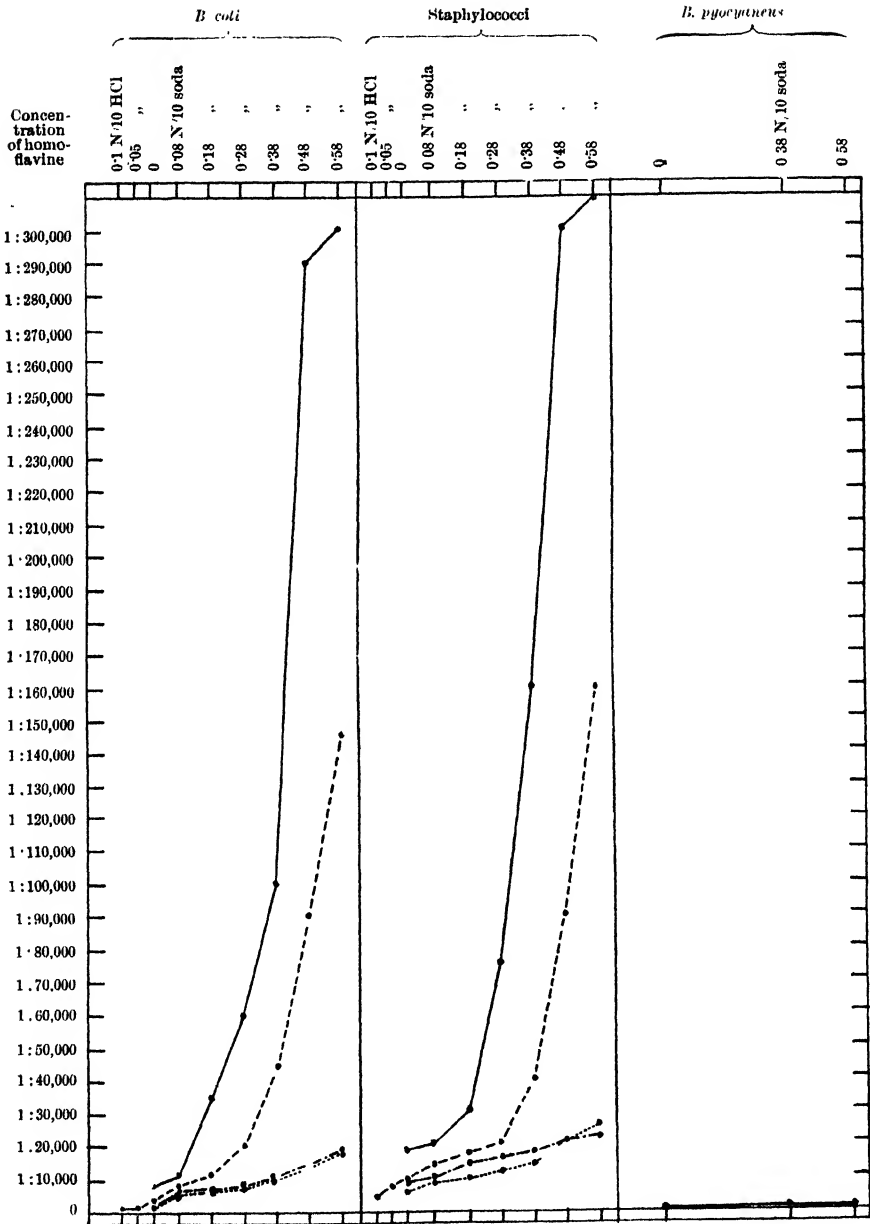


CHART I. Showing the influence of the quantity of nutrient material and change of reaction on the concentration of homoflavine necessary to cause inhibition of growth on agar during 48 hours' incubation at 37° C.

# Action of Dyes on Bacteria

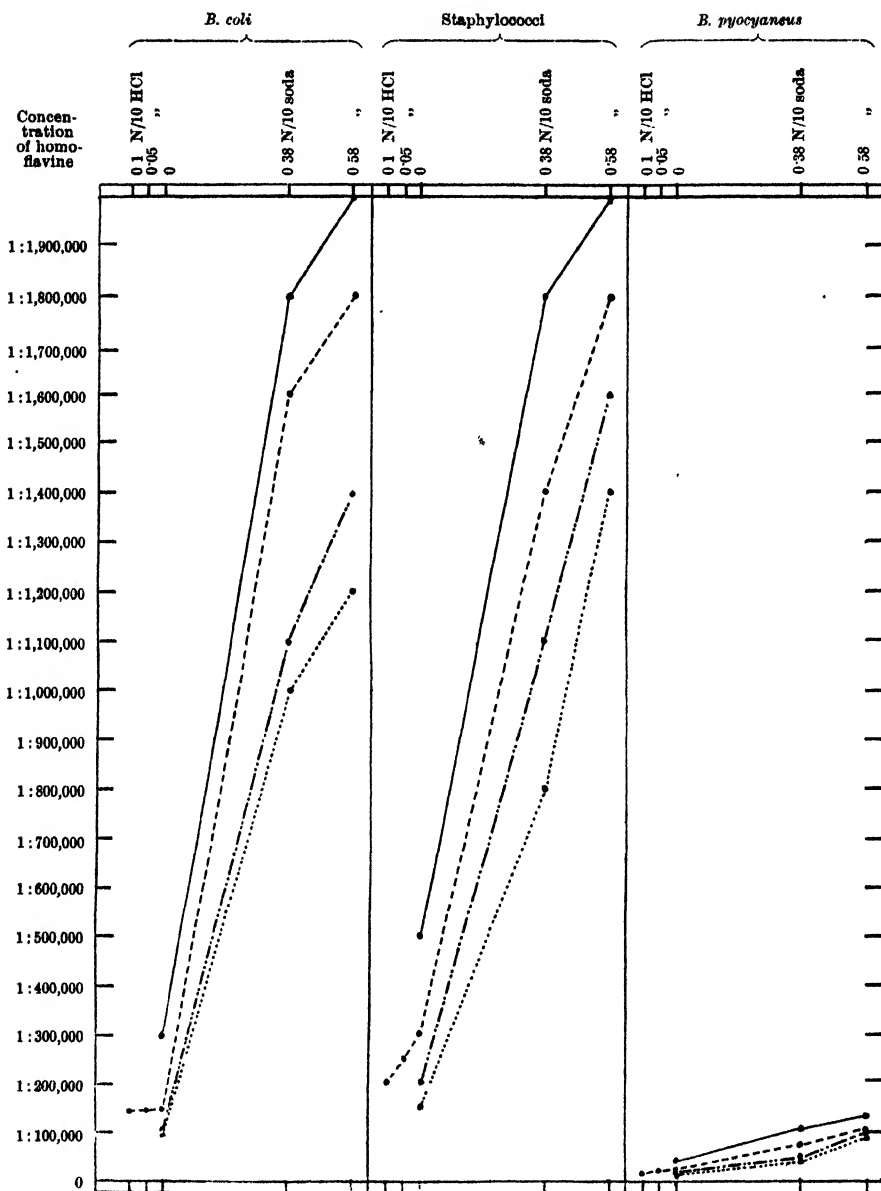


CHART II. Showing the influence of the quantity of nutrient material and change of reaction on the concentration of homoflavine necessary to cause inhibition of growth in meat extract during 48 hours' incubation at 37° C.

— 0.5 c.c. meat extract      - · - · - 1.5 c.c. meat extract  
 - - - 1.0 c.c. " "      · · · · · 2.0 c.c. " "

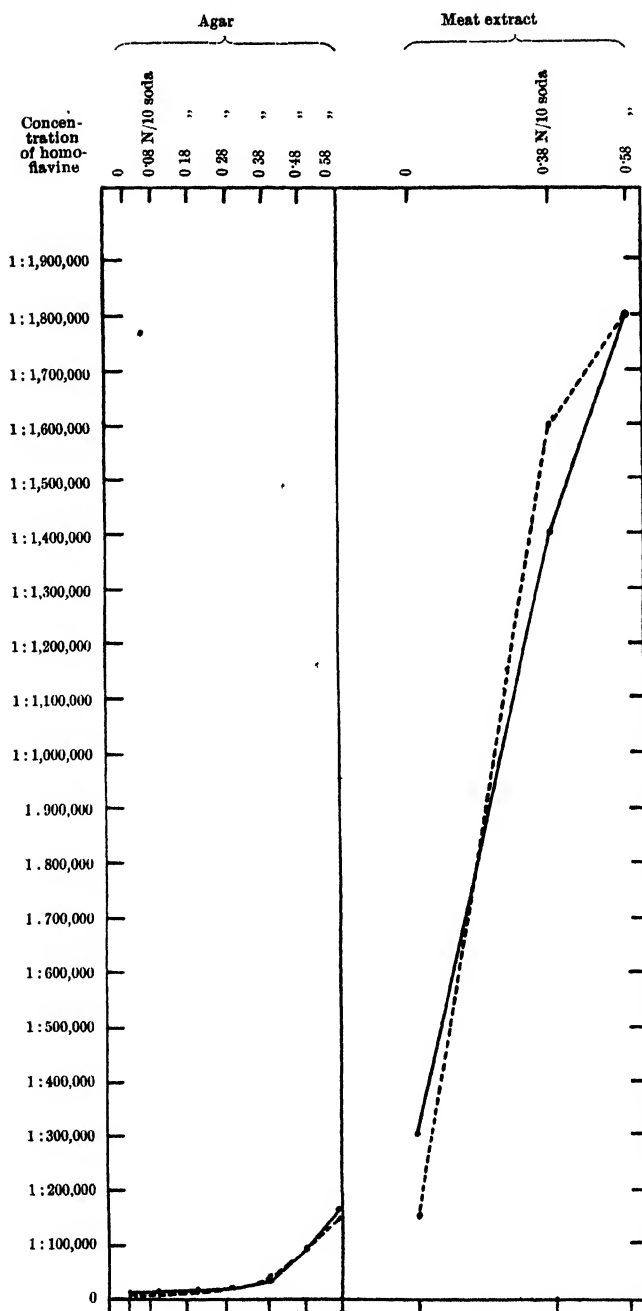


CHART III. Illustrating the differences in concentrations of homoflavine necessary to inhibit the growth of staphylococci and *B. coli* during 48 hours' incubation at 37° C. when growing on agar and in meat extract, when each tube contains 1 c.c. of nutrient material.

--- *B. coli*

— staphylococci



## *Action of Dyes on Bacteria*

It will be noticed that the curves for *B. coli* and staphylococci are very similar. In this connection it is of interest to note that these organisms multiply at much the same rate on *meat extract* (without the addition of homoflavine) as shown by counting the colonies on agar subcultures made at different periods of growth. On the other hand *B. pyocyaneus* multiplies much more rapidly.

The action of homoflavine on *B. pyocyaneus* on agar is very little influenced by the addition of soda, and not very greatly influenced in the meat extract.

Chart III has been constructed to bring out more clearly the remarkable difference between the actions of homoflavine in agar and in meat extract when acting on *B. coli* and staphylococci.

In order to ascertain whether the striking differences between the inhibiting concentrations on agar and in meat extract were due to the prevalence of aerobic conditions in the agar plates and partial anaerobic conditions in the neutral meat extract tubes, cultures were made in aerated meat extract and boiled meat extract under paraffin. The results were almost identical. The growth of the staphylococci was inhibited in each series at a concentration of 1 : 550,000, of *B. coli* at 1 : 250,000 and of *B. pyocyaneus* of about 1 : 40,000.

A series of experiments were carried out to ascertain the influence of reaction on the growth of these organisms on the media described with varying quantities of meat extract, replacing the homoflavine solution with distilled water. The results are given in the following table.

	Agar			Meat extract		
	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>	<i>B. coli</i>	Staphylo- cocci	<i>B. pyo- cyaneus</i>
	2 c.c. meat extract	2 c.c. meat extract	2 c.c. meat extract	1 c.c. meat extract	1 c.c. meat extract	1 c.c. meat extract
	1 " "	1 " "	1 " "			
	0.5 " "	0.5 " "	0.5 " "			
N/10 soda						
2.5 c.c.	- - -	- - -	- - -	-	-	-
2.25	- - -	+ - -	- - -			
2.0	+ - -	+ + -	+ + +			
1.9	+ - -	+ + -	+ + +			
1.8	- - -	+ + -	+ + +			
1.7	+ + -	+ + -	+ + +		+	+
1.6	+ + +	+ + +	+ + +	-	+	+
1.5	+ + +	+ + +	+ + +	-	+	+
1.4	+ + +	+ + +	+ + +	+	+	+
N/10 HCl						
0.1 c.c.	+ + +	+ + +	+ + +	+	+	+
0.125	+ + +	+ + +	+ + +	+	+	+
0.150	+ + -	+ + -	+ + -	+	-	+
0.175	+ + -	+ + -	+ - -	-	-	-
0.2	+ - -	+ - -	+ - -			
0.3	- - -	- - -	- - -			

+ = growth after 48 hours' incubation. - = no growth.

This table shows that the quantity of acid or alkali which is necessary to add in order to inhibit growth depends to some extent on the quantity of nutrient material present. While on the alkaline side the range is considerable, the range on the acid side is very small, but it may be increased to some extent by increasing the concentration of meat extract. With 2.5 c.c. of meat extract growth of staphylococci occurs in the fluid medium when 0.3 c.c. of N/10 HCl is added, and with 5 c.c. of meat extract when 0.8 c.c. of N/10 HCl is present.

Such small quantities of N/10 soda as greatly influence the action of homoflavine do not of themselves appear to have any influence on the growths, when these are compared with neutral controls.

In the case of *B. coli* and *B. pyocyaneus* the organisms are very short, rounded and almost coccus-like when growing on the acid medium, and very long, thin and irregular when growing on the higher concentrations of soda.

*The effect of varying the proportion of agar.*

To determine whether the quantity of agar employed has any influence on the concentration of homoflavine necessary to inhibit the growth of *B. coli* and staphylococci, experiments with media of the following composition were carried out.

	Meat extract	Agar (2%)	N/10 soda	Distilled water	Solution of homoflavine
A	1.0	1.0	0.08	2.42	0.5
B	1.0	2.0	0.08	1.42	0.5
C	1.0	3.0	0.08	0.42	0.5

After 48 hours' incubation at 37° C. it was seen that different concentrations of homoflavine were necessary to inhibit growth in these three media.

	Growth inhibited at a con- centration of	
	<i>B. coli</i>	Staphylococci
A	1: 14,000	1: 30,000
B	1: 9,000	1: 14,000
C	1: 7,000	1: 9,000

Homoflavine does not seem to enter into strong combination with the agar, for if divided plates are made according to Churchman's (1912) method, having a solution of homoflavine mixed with the agar on one side and not on the other, and the partition walls are removed immediately after the agar has set, and subsequently emulsions of organisms are stroked across the medium at right angles to the dividing line, the plain medium becomes coloured for a short distance beyond the dividing line and colonies at a considerable distance beyond the line take up the stain and become yellow.

The diffusion of homoflavine and its consequences can be well illustrated in another manner. Two parallel streaks of plain agar (2%), one broad and the other narrow, are made on the bottom of a Petri dish and allowed to set, and then medium of the composition of B in the experiment just quoted is poured into the dish so as to cover the streaks and allowed to set. Subsequently emulsions of *B. coli* and staphylococci are stroked across the plate at right angles to the agar streaks. At a concentration of 1: 9,000 a few colonies of

*B. coli* grow immediately over the streaks, but not elsewhere, though when the concentration decreases to 1:17,000 colonies appear wherever the emulsion was spread, but most thickly over the streaks. Similarly colonies of staphylococci grow over the broad streak only at a concentration of 1:10,000, over both streaks at 1:14,000 and wherever the emulsion was spread at a concentration of 1:14,000 and wherever the emulsion was spread at a concentration of 1:20,000, showing that the homoflavine diffuses into the plain agar streaks, and consequently so lowers the concentration over them that the organisms can grow though inhibited elsewhere (Plate I, fig. 21).

Morphologically the *B. coli* from the colonies growing over the streaks at a concentration of 1:9,000 are very long and irregular, but those growing in the same situation at a concentration of 1:17,000 are much shorter and more normal in appearance. The change in morphology from very long to short normal forms may be traced in the divided plates just mentioned when passing from the extremity of the stroke of emulsion on the side containing the homoflavine to the opposite extremity.

Strong concentrations of homoflavine (1:1,000) seem to cause the agar to set very firmly and to lessen the exudation of water from it.

*The effects of salt and of peptone on agar cultures.*

In order to ascertain the influence of the presence of 0.75 % salt and 1 % peptone on agar cultures the following series A, B, C, D of experiments were carried out.

The members of each series contained					There were added in						
					Series A	Series B		Series C		Series D	
	Meat extract	Agar	N/10 HCl	N/10 soda	Dis-tilled water	15% salt	Dis-tilled water	20% peptone	Dis-tilled water	20% peptone in 15% salt	Dis-tilled water
1.	1.0	2.0	0.4	—	1.6	0.25	1.35	0.25	1.35	0.25	1.35
2.	1.0	2.0	0.3	—	1.7	0.25	1.45	0.25	1.45	0.25	1.45
3.	1.0	2.0	0.2	—	1.8	0.25	1.55	0.25	1.55	0.25	1.55
4.	1.0	2.0	0.1	—	1.9	0.25	1.65	0.25	1.65	0.25	1.65
5.	1.0	2.0	—	—	2.0	0.25	1.75	0.25	1.75	0.25	1.75
6.	1.0	2.0	—	1.0	1.0	0.25	0.5	0.25	0.5	0.25	0.5
7.	1.0	2.0	—	1.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25
8.	1.0	2.0	—	1.75	0.25	0.25	0.0	0.25	0.0	0.25	0.0
9.	1.0	2.0	—	0.2 (N)	1.8	0.25	1.55	0.25	1.55	0.25	1.55
10.	1.0	2.0	—	0.25 (N)	1.75	0.25	1.5	0.25	1.5	0.25	1.5

After 48 hours' incubation at 37° C. the results were as follows:

	<i>B. coli</i> Series				Staphylococci Series				<i>B. pyocyaneus</i> Series			
	A	B	C	D	A	C	B	D	A	B	C	D
1.	0	0	0	+	0	0	0	×	0	0	0	0
2.	0	0	×	+	0	0	×	+	0	0	0	0
3.	×	+	+	+	×	+	+	+	0	×	+	+
4.	+	+	+	+	+	+	+	+	+	+	+	+
5.	+	+	+	+	+	+	+	+	+	+	+	+
6.	+	+	+	+	+	+	+	+	+	+	+	+
7.	+	+	+	+	+	+	+	+	+	+	+	+
8.	+	+	+	+	+	+	+	+	+	+	+	+
9.	+	×	0	0	+	+	0	×	+	+	0	0
10.	+	0	0	0	+	+	0	0	+	0	0	0

+ = good growth, × = slight growth, and 0 = no growth.

In the case of *B. coli* the presence of salt or peptone diminishes the range of growth on the alkaline side, and slightly increases it on the acid side, but the presence of both distinctly increases it on the acid side. In the case of staphylococcus the presence of salt has little effect, but peptone decreases the range on the alkaline side, and the presence of both increases the range on the acid side. In the case of *B. pyocyaneus* the range on the acid side is slightly increased and on the alkaline side distinctly decreased by both salt and peptone alone or combined.

*The action of homoflavine on cultures of various ages.*

Cultures were grown on meat extract 1 c.c., N/10 soda 0.08 c.c., water 3.5 c.c. for one, three and ten days respectively. At the expiration of these periods 0.5 c.c. of a solution of homoflavine was added, and the tubes returned to the incubator. After one, two and eight days' incubation a loopful from each tube was sown on agar, and the result recorded after two days at 37° C.

Cultures 24 hours old

Concentration of homoflavine for <i>B. coli</i> and staphylococci	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine			After 8 days' contact with homoflavine		
	<i>B. coli</i>	Staphylococci	Concentration for <i>B. pyocyaneus</i>	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
: 50,000	0	0	1: 550	0	0	0	0	few	0	0
: 100,000	numerous	many	1: 750	0	many	0	0	numerous	0	0
: 150,000	"	numerous	1: 1,000	0	"	0	0	"	0	0
: 200,000	"	"	1: 2,000	numerous	"	0	few	"	0	0
: 250,000	"	"	1: 10,000	"	numerous	0	numerous	"	0	numerous

Cultures three days old

Concentration for <i>B. coli</i> and staphylococci	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine			After 8 days' contact with homoflavine		
	<i>B. coli</i>	Staphylococci	Concentration for <i>B. pyocyaneus</i>	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 50,000	numerous	few	1: 550	0	0	0	0	0	0	0
1: 100,000	"	"	1: 750	0	few	0	0	numerous	0	0
1: 150,000	"	numerous	1: 1,000	few	"	0	0	"	0	0
1: 200,000	"	"	1: 2,000	"	"	0	0	"	0	0
1: 250,000	"	"	1: 10,000	many	many	few	few	"	0	0

Cultures 10 days old

Concentration for <i>B. coli</i> and staphylococci	After 24 hours' contact with homoflavine				After 48 hours' contact with homoflavine		
	<i>B. coli</i>	Staphylococci	Concentration for <i>B. pyocyaneus</i>	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 50,000	few	0	1: 550	0	0	0	0
1: 100,000	numerous	0	1: 750	0	0	0	0
1: 150,000	"	0	1: 1,000	numerous	few	0	0
1: 200,000	"	few	1: 2,000	"	"	0	0
1: 250,000	"	"	1: 10,000	"	"	0	0

These experiments, which have not been repeated, seem to show that in cultures during the rapidly growing stages staphylococci are killed more readily than *B. coli* by the prolonged action of certain strengths of the homoflavine. In the case of *B. coli* after a period of inhibition multiplication may occur. The older cultures of all these organisms seem to be the most susceptible to the prolonged action of homoflavine.

If cultures on such a medium (the homoflavine being replaced by distilled water) be examined by means of dilutions in plate cultures it will be found that at the end of 24 hours' incubation great multiplication of the organisms has taken place, and that by the third day the growth in the case of *B. coli* and staphylococci has nearly reached its maximum. Subsequently multiplication ceases, and by the tenth day a great reduction in numbers has taken place. *B. pyocyaneus* reaches its maximum very early, and active multiplication has ceased by the third day.

It is evident from these experiments that the results obtained by adding solutions of homoflavine to actively growing or declining cultures are very different from those obtained in previous experiments in which relatively small numbers of organisms were added to media already containing homoflavine.

The experiments which have been quoted show that the action of homoflavine is very greatly influenced by the reaction of the medium, the quantity of nutrient substance, the presence of agar, and the age of the culture; in fact any alteration in the composition of the medium, or the proportion of the ingredients, affects the results obtained to a greater or less degree. (See also pp. 16, 18).

#### *Comparison of Acriflavine and Homoflavine.*

The effects of acriflavine and homoflavine in meat extract cultures have not been compared, but some comparative experiments in ox serum, sterilised by heating to 55° C., and in 0.7 % peptone water, were made.

In each tube were placed 0.8 c.c. of serum or 0.8 c.c. of 0.7 % peptone water, 0.1 c.c. of a solution of the dye and 0.1 c.c. of a dilution (staphylococci 1 : 20,000, *B. coli* 1 : 10,000) in 0.75 % salt solution of a 24 hours' peptone water culture of the organism. Fortunately the two dilutions contained almost the same number of organisms. Control tubes without the dye were sown at the same time.

The following table (p. 15) shows the results of this experiment, the figures indicating the numbers of organisms growing in cultures on agar made with one standard loopful (0.01 c.c.), after dilution if this seemed necessary.

The peptone solution was distinctly acid, 5 c.c. requiring 0.275 N/10 soda to neutralise it to neutral red, and the serum distinctly alkaline, 5 c.c. requiring 0.75 N/10 HCl to neutralise it.

These experiments show that the actions of the two dyes on staphylococci and *B. coli* respectively are very similar, but that while the staphylococci are

Concentration of dye	Cultures in 0.7% peptone										Cultures in serum			
	Acridine					Homoflavine					Acridine		Homoflavine	
	5	23	51	73 hrs.	5	23	51	73 hrs.	5	23	51	73 hrs.	5	23
1: 50,000	-	0	0	0	-	0	0	0	-	0	0	0	-	0
1: 100,000	1	0	0	0	16	0	0	0	1	0	0	0	3	0
1: 125,000	-	0	0	0	-	0	0	0	-	0	0	0	-	0
1: 150,000	6	0	0	0	16	0	0	0	20	262	7,250	365,000	1	6
1: 175,000	-	0	0	0	-	0	0	0	-	750	-	-	-	1
1: 200,000	7	0	0	0	14	0	0	0	33	51,000	640,000	-	16	973,000
1: 250,000	-	89,000	5,128,000	-	-	0	0	0	-	982,000	1,932,000	-	-	1,148,000
Immediate														
Control 30	68	12,300,000	-	-	-	Control 32	225	2,120,000	-	-	-	-	-	-

Concentration of dye	Cultures in 0.7% peptone										Cultures in serum			
	Acridine					Homoflavine					Acridine		Homoflavine	
	5	23	51	73 hrs.	5	23	51	73 hrs.	5	23	51	73 hrs.	5	23
1: 50,000	-	0	0	0	-	0	0	0	-	0	0	0	-	0
1: 100,000	2	0	0	0	2	15	5,500	17,760	0	0	0	0	0	0
1: 125,000	-	90,880	-	-	-	50	-	39,360	-	0	0	0	0	0
1: 150,000	2	220,000	402,000	-	1	104,800	-	148,000	1	0	0	0	0	0
1: 175,000	-	400,000	-	-	-	-	-	-	-	0	0	0	0	0
1: 200,000	24	843,200	-	-	4	302,000	-	1,180,000	1	0	0	0	0	0
1: 250,000	-	1,392,000	3,208,000	-	-	-	-	-	-	0	0	0	0	0
Immediate														
Control 31	48	4,048,000	10,016,000	-	-	Control 37	427	7,112,000	-	-	-	-	-	-

0 = no growth in subculture, - = subculture not made.

more susceptible when growing in peptone water the *B. coli* are more susceptible when growing in serum. In the lower concentrations the organisms in many cases at first seemed to decrease in numbers and then to increase.

### Quinone.

Several series of experiments, comparable with those made with homo-flavine, were carried out with quinone.

#### *The effects of varying the reaction of the medium.*

To test the effect of alterations in the reaction of the medium experiments were made on agar and in meat extract. In the former series the agar was melted, and the soda and water added. It was then cooled to 45° C., the solution of quinone added, the contents of the tube thoroughly mixed and the medium poured into plates and allowed to set.

	Meat extract	N/10 soda	N/10 HCl	Agar	Water	Solution of quinone
1.	1.0	—	0.15	2.0	1.35	0.5
2.	1.0	—	0.1	2.0	1.4	0.5
3.	1.0	—	0.05	2.0	1.45	0.5
4.	1.0	—	—	2.0	1.5	0.5
5.	1.0	0.08	—	2.0	1.42	0.5
6.	1.0	0.18	—	2.0	1.32	0.5
7.	1.0	0.28	—	2.0	1.22	0.5
8.	1.0	0.38	—	2.0	1.12	0.5
9.	1.0	0.48	—	2.0	1.02	0.5
10.	1.0	0.58	—	2.0	0.92	0.5

The meat extract series was similar in all respects except that 2.0 c.c. of water were substituted for the 2.0 c.c. of agar. The solution of quinone was added when the medium had cooled after sterilisation.

The results are given in the following table, which shows the concentration of quinone necessary in order to completely inhibit growth for 48 hours when the agar and meat extract contain 1 c.c. of nutrient material.

	Agar			Meat extract		
	<i>B. coli</i>	<i>Staphylo- cocci</i>	<i>B. pyo- cyaneus</i>	<i>B. coli</i>	<i>Staphylo- cocci</i>	<i>B. pyo- cyaneus</i>
N/10 HCl						
1. = 0.15	1: 100,000	1: 45,000	1: 30,000	—	—	—
2. = 0.1	1: 90,000	1: 40,000	1: 20,000	—	1: 120,000	1: 55,000
3. = 0.05	1: 70,000	1: 50,000	1: 25,000	1: 120,000	1: 60,000	1: 70,000
4. = 0	1: 90,000	1: 70,000	1: 25,000	1: 90,000	1: 50,000	1: 7,500
N/10 soda						
5. = 0.08	1: 95,000	1: 70,000	1: 30,000	1: 110,000	1: 60,000	1: 10,000
6. = 0.18	1: 90,000	1: 55,000	1: 30,000	1: 90,000	1: 40,000	1: 15,000
7. = 0.28	1: 75,000	1: 45,000	1: 27,000	1: 40,000	1: 30,000	1: 15,000
8. = 0.38	1: 60,000	1: 30,000	1: 25,000	1: 25,000	1: 20,000	1: 12,500
9. = 0.48	1: 50,000	1: 20,000	1: 20,000	1: 17,500	1: 25,000	1: 10,000
10. = 0.58	1: 45,000	1: 15,000	1: 15,000	1: 10,000	1: 25,000	1: 7,500

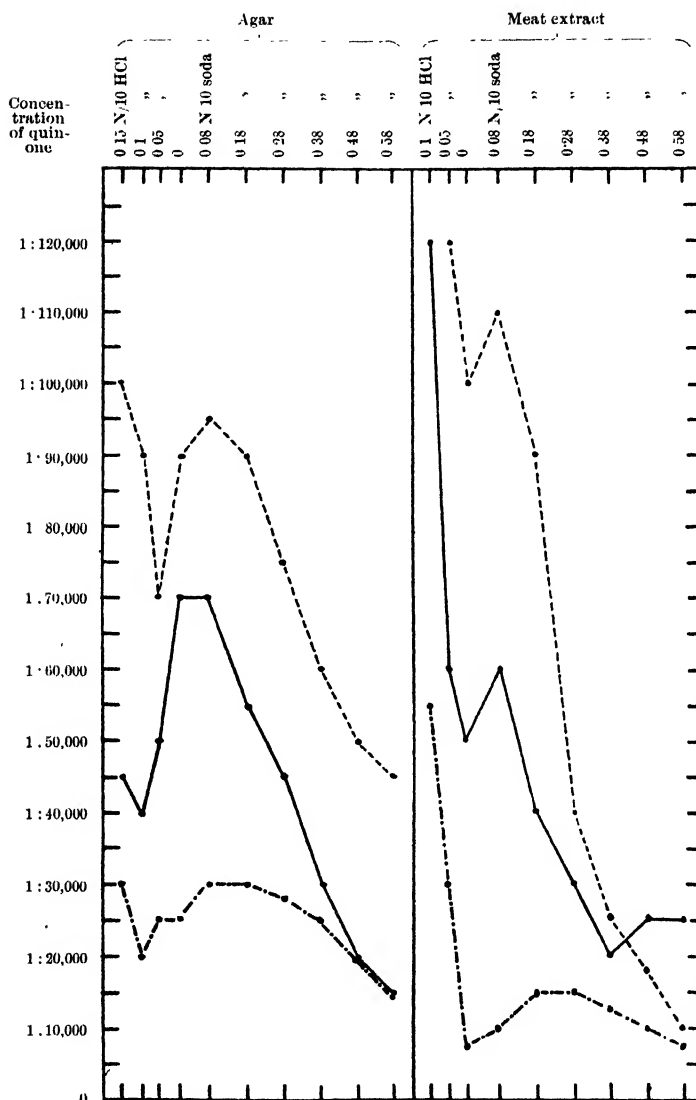


CHART IV. Showing the influence of change of reaction on the concentration of quinone necessary to cause inhibition of growth during 48 hours at 37° C. on agar and in meat extract.

----- *B. coli* ————— staphylococci  
 - · - · - *B. pyocyaneus*



## Action of Dyes on Bacteria

It will be seen by comparing Charts I, II, III, and IV that while the efficiency of homoflavine increases with the increase of alkalinity the efficiency of quinone diminishes. While homoflavine is most active in an alkaline solution, quinone is most active at or near neutrality to neutral red. Further there is not so marked a difference between the concentrations of quinone necessary to produce inhibition in agar and in meat extract as there is in the case of homoflavine.

### The effect of varying the proportion of nutrient material.

The efficiency of quinone decreases as the concentration of nutrient material increases as shown by the following experiment on agar.

	Composition of medium					Concentration of quinone required to inhibit		
	Meat extract	Agar	N/10 soda	Water	Solution of quinone	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1.	0.5	2.0	0.04	2.0	0.5	1: 150,000	1: 110,000	1: 15,000
2.	1.0	2.0	0.08	1.5	0.5	1: 85,000	1: 65,000	1: 12,000
3.	1.5	2.0	0.12	1.4	0.5	1: 60,000	1: 35,000	1: 10,000
4.	2.0	2.0	0.16	1.3	0.5	1: 50,000	1: 25,000	1: 8,000

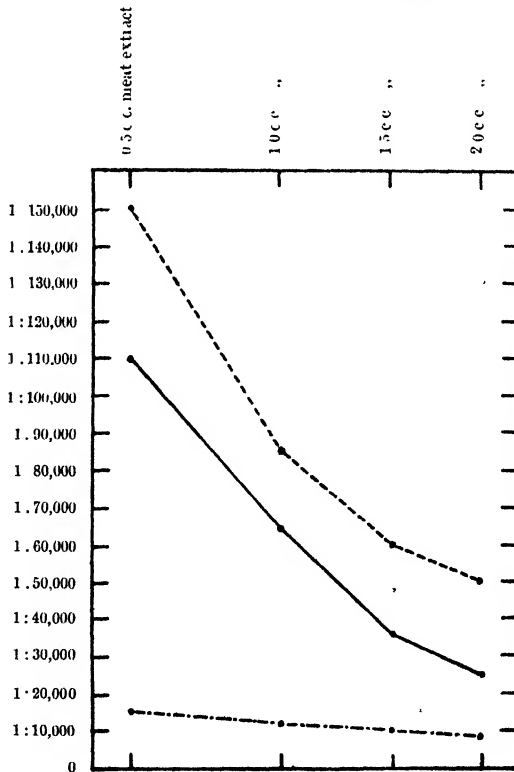


CHART V. Showing the concentrations of quinone necessary to cause inhibition of growth on agar during 48 hours at 37° C. in the presence of different quantities of nutrient material.

----- *B. coli*                      ——— staphylococci  
 - · - · - *B. pyocyaneus*

*The effect of varying the proportion of agar.*

The efficiency of quinone is only slightly influenced by the quantity of agar present.

	Composition of medium					Concentration of quinone required to inhibit the growth of staphylococci
	Meat extract	Agar	N/10 soda	Water	Solution of quinone	
1.	1.0	1.0	0.08	2.42	0.5	1 : 60,000
2.	1.0	2.0	0.08	1.42	0.5	1 : 70,000
3.	1.0	3.0	0.08	0.42	0.5	1 : 80,000

*The effect of heat on the action of quinone.*

The action of quinone, unlike the action of homoflavine, is greatly influenced by heat, as shown in the following experiments. In one series all the ingredients were mixed and the medium boiled for five minutes before pouring

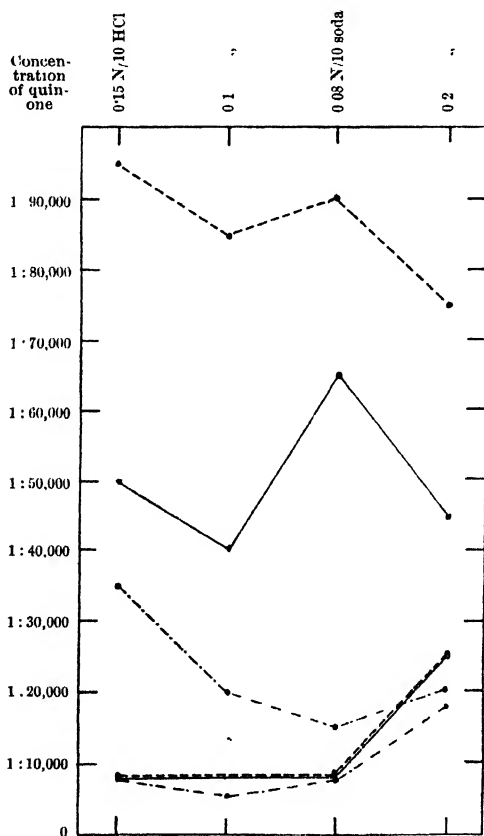


CHART VI. Showing the influence of boiling on the action of quinone. Upper lines indicate unheated series, lower lines boiled series.

----- *B. coli*                      ——— staphylococci  
 - . - . - *B. pyocyaneus*

## Action of Dyes on Bacteria

the plates, while in the other the ingredients without the quinone were first mixed and boiled. After cooling to 45° C. the solution of quinone was added and plates immediately poured.

Composition of media						
	Meat extract	N/10 HCl	N/10 soda	Agar	Water	Solution of quinone
A.	1.0	0.15	—	2.0	1.35	0.5
B.	1.0	0.05	—	2.0	1.45	0.5
C.	1.0	—	0.08	2.0	1.42	0.5
D.	1.0	—	0.2	2.0	1.3	0.5

Concentration of quinone required to inhibit growth							
Unheated quinone solution				Heated quinone solution			
	A	B	C	D	A	B	C
<i>B. coli</i>	1: 95,000	1: 85,000	1: 90,000	1: 75,000	1: 7,500	1: 7,500	1: 7,500
Staph.	1: 50,000	1: 40,000	1: 65,000	1: 45,000	1: 7,500	1: 7,500	1: 7,500
<i>B. pyocyaneus</i>	1: 35,000	1: 15,000	1: 15,000	1: 15,000	1: 7,500	1: 3,000	1: 7,500

### The action of quinone on cultures of various ages.

Cultures made in meat extract 1.0 c.c., N/10 soda 0.8 c.c., and water 3.5 c.c., were incubated at 37° C. for one, three and ten days respectively. At the expiration of these periods 0.5 c.c. of a solution of quinone was added to each, and the tube returned to the incubator. 24 and 72 hours later one standard loopful was sown from each culture on agar, and incubated for 48 hours.

Cultures one day old							
Concentration of quinone	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	<i>B. coli</i>	Staphylococci	Concentration of quinone	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 10,000	numerous	0	1: 1,000	0	numerous	0	0
1: 25,000	"	numerous	1: 2,500	0	"	numerous	0
1: 50,000	"	"	1: 5,000	numerous	"	"	numerous
1: 75,000	"	"	1: 7,500	"	"	"	"

Cultures three days old							
Concentration of quinone	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	<i>B. coli</i>	Staphylococci	Concentration of quinone	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
1: 10,000	numerous	0	1: 1,000	0	numerous	0	0
1: 25,000	"	0	1: 2,500	0	"	0	0
1: 50,000	"	0	1: 5,000	numerous	"	0	numerous
1: 75,000	"	0	1: 7,500	"	"	numerous	"
1: 100,000	"	0	1: 10,000	"	"	"	"

Cultures ten days old							
Concentration of quinone	After 24 hours' contact with quinone				After 72 hours' contact with quinone		
	<i>B. coli</i>	Staphylococci	Concentration of quinone	<i>B. pyocyaneus</i>	<i>B. coli</i>	Staphylococci	<i>B. pyocyaneus</i>
10,000	0	0	1: 1,000	0	numerous	0	0
25,000	few	0	1: 2,500	0	"	0	0
50,000	numerous	0	1: 5,000	numerous	"	0	numerous
75,000	"	0	1: 7,500	"	"	0	"
100,000	"	0	1: 10,000	"	"	0	"

In the case of staphylococci it will be noticed that the older the culture the more affected it is by treatment with quinone. In the case of *B. coli* this phenomenon is not so evident. *B. pyocyaneus* is equally affected at all ages.

As in the case of homoflavine the results obtained by adding the solution of quinone to growing cultures are very different from those obtained when relatively small numbers of organisms are added to media containing a solution of quinone.

**The influence of numbers of organisms and of gelatin on the effects of homoflavine and of quinone.**

*The influence of numbers in the initial dose of organisms.*

Certain experiments illustrating the influence of variations in the numbers of the organisms in the initial dose on the effects of homoflavine and quinone were carried out simultaneously and are best considered in conjunction.

The culture medium (neutral to neutral red) consisted of meat extract 1.0 c.c., soda 0.8 c.c., water 3.42 c.c. and dye 0.5 c.c. In series A the concentration of homoflavine was 1 : 100,000 and in series B 1 : 250,000, in series C the concentration of quinone was 1 : 20,000, and in series D 1 : 50,000. Each series consisted of seven tubes to each of which one drop of an emulsion in distilled water of staphylococci from an agar culture grown at 37° C. for 24 hours was added. The first tube received a drop of a strong emulsion, the next of an emulsion of 1/10 strength, and the others of 1/100, 1/1000, 1/10,000, 1/100,000, 1/1,000,000 strengths. The organisms present in these tubes were estimated immediately by sowing standard loops (0.01 c.c.) of the cultures in melted agar and pouring plates, and in the same way the numbers were estimated, using dilutions when necessary, after 24, 48, 72 and 96 hours' cultivation at 37° C.

It will be seen that in the controls very active multiplication took place within 24 hours in all the cases tested.

In a concentration of 1 : 100,000 homoflavine, series A, a few of the organisms remained alive in tube 1, to which the greatest numbers were added, but none appeared to remain alive in the other tubes to which smaller numbers were added. At a concentration of 1 : 250,000 of homoflavine, series B, multiplication of the organisms occurred rapidly in tube 1, more slowly in tube 2, and some multiplication, after an initial diminution, in tubes 3 and 5. In the latter case the organisms were so few after 24 hours' incubation that no colonies appeared in the subculture made with 0.01 c.c. of the culture. In the other tubes no organisms appeared to survive. It seems probable therefore that, whereas in tubes 4, 6 and 7 all the organisms were destroyed in the stage when diminution in numbers is occurring, in tube 5 one or more survived, became accustomed to the conditions and subsequently multiplied.

A concentration of 1 : 20,000 of quinone, series C, inhibited growth in all cases, while with a concentration of 1 : 50,000, series D, after a great initial

*Action of Dyes on Bacteria*

diminution rapid growth occurred in tube 1, but no colonies ever appeared in subcultures from the other tubes.

No of tube	Dilution of cocci	Homoflavine		Quinine		Homoflavine			Quinine					
		Series A		Series C		Series B			Series D					
		Immed count per loop	24	48	24	48	24	48	72	96	24	48	72	96 hrs.
1.	full	83,500	57	61	0	0	752,000	4,848,000	5,424,000	5,968,000	58	5,131,000	8,352,000	8,432,000
2.	1/10	7,904	0	0	0	0	9,100	1,758,000	2,368,000	2,784,000	0	0	0	0
3.	1/100	872	0	0	0	0	29	23,309	1,536,000	2,640,000	0	0	0	0
4.	1/1,000	94	0	0	0	0	0	0	0	0	0	0	0	0
5.	1/10,000	7	0	0	0	0	0	8	10,080	2,576,000	0	0	0	0
6.	1/100,000	1	0	0	0	0	0	0	0	0	0	0	0	0
7.	1/1,000,000	0.8	0	0	0	0	0	0	0	0	0	0	0	0
Controls		Immediate		24 hours										
	1/10		7,904		6,620,000									
	1/1,000		872		5,744,000									
	1/10,000		7		5,166,000									
	1/1,000,000		0.8		2,800,000									

Several other experiments of this type gave similar results, showing that the concentration of the compound necessary to inhibit growth is greatly

influenced by the initial number of organisms present. Small numbers of staphylococci seem to be able to overcome the influence of homoflavine more easily than the influence of quinone.

*The influence of gelatin.*

Several series of experiments on the influence of gelatin on the effects of homoflavine and of quinone were carried out. The first series quoted is intended to illustrate the influence of gelatin in the presence of strong concentrations of these compounds.

In this experiment small quantities of gelatin, agar or albumin were added in some of the tubes, while other tubes in the series acted as controls. All were made neutral to neutral red. The tubes were sterilised by boiling and the solution of the compound, sufficient to make a final concentration of 1 : 1,000, the albumin and a drop of an emulsion of staphylococci added after cooling.

No of tube	Meat extract	Salt solution (3.75 %)	N/10 soda	N/10 HCl	Gelatin (20 %)	Agar (2 %)	Egg albumin	Water	Solution of compound
1.	1.0	1.0	0.08	—	—	—	—	2.42	0.5
2.	1.0	1.0	0.17	—	1.0	—	—	1.23	0.5
3.	—	1.0	0.09	—	1.0	—	—	2.41	0.5
4.	1.0	1.0	0.08	—	—	0.5	—	1.92	0.5
5.	—	1.0	—	—	—	0.5	—	3.0	0.5
6.	—	1.0	—	—	—	—	—	3.5	0.5
7.	1.0	1.0	0.08	—	—	—	0.5	1.92	0.5
8.	—	1.0	—	—	—	—	0.5	3.0	0.5
9.	—	1.0	—	—	—	—	0.5	3.5	—
10.	—	1.0	—	—	—	—	—	4.0	—
11.	—	1.0	—	0.1	—	—	—	3.9	—
12.	—	1.0	1.0	—	—	—	—	3.0	—

Immediately after the introduction of the organisms and after 1, 5½ and 24 hours' incubation at 37° C. standard loopfuls were sown in agar plates, the colonies on which were counted after 48 hours' incubation, with the following results.

Tube	Homoflavine			Quinone		
	1 hour	5.5 hours	24 hours	1 hour	5.5 hours	24 hours
1.	280	0	0	0	0	0
2.	3,264	168	0	0	0	0
3.	5,886	1,136	0	0	0	0
4.	488	1	0	0	0	0
5.	4,000	37	0	0	0	0
6.	2,656	360	0	0	0	0
7.	1,072	0	0	0	0	0
8.	1,696	0	0	0	0	0
Control tubes						
	1 hour	5.5 hours	24 hours			
9.	7,200	6,016	4,448			
10.	5,984	4,624	0			
11.	26	23	2			
12.	7,616	1,632	0			

The immediate cultures yielded an average of 9950 organisms per loop.

It will be seen that a rapid diminution in the number of organisms took

place in the presence of 1:1,000 homoflavine in tube 1, containing meat extract only, and in tube 4, containing meat extract and agar. The numbers diminished much less speedily in tube 2 containing meat extract and gelatin, tube 3 containing gelatin only, tube 5, containing agar without meat extract, and tubes 7 and 8, containing albumin. The numbers also diminished slowly in tube 6 containing salt solution. On the other hand in a concentration of 1:1,000 neither gelatin, agar, albumin or salt solution seems to retard the effects of quinone.

In the control tubes 10, 11, 12, containing neutral, acid and alkaline salt solution, very few or no living organisms were found after 24 hours' incubation, but numerous colonies were present in subcultures made at that time from tube 9, containing albumin.

The influence of various quantities of gelatin, with and without meat extract, was studied in another series of experiments.

Tubes containing media of the following composition were prepared and sterilised by boiling. The solution of the compound and one drop of a staphylococcus emulsion were added when cool, and the cultures incubated at 37° C. Immediate counts showed that the cultures contained an average of 2,016 organisms per loop.

		Meat extract	Gelatin (20 %)	N/10 soda	Water	Solution of compound
Controls	1.	—	2.0	0.18	2.82	—
	2.	—	1.0	0.09	3.91	—
	3.	—	0.5	0.045	4.45	—
	4.	—	2.0	0.18	2.32	0.5
	5.	—	1.0	0.09	3.41	0.5
	6.	—	0.5	0.045	3.95	0.5
Controls	7.	1.0	2.0	0.26	1.74	—
	8.	1.0	1.0	0.17	2.83	—
	9.	1.0	0.5	0.125	3.375	—
	10.	1.0	2.0	0.26	1.24	0.5
	11.	1.0	1.0	0.17	2.33	0.5
	12.	1.0	0.5	0.125	2.875	0.5

Of numbers 4, 5, 6, 10, 11 and 12 four tubes were prepared, two (*a* and *b*) receiving solution of homoflavine of such strength as to make concentrations of 1:1,000 and 1:10,000 and two (*c* and *d*) solutions of quinone to make concentrations of 1:1,000 and 1:10,000. The organisms present in the cultures were counted by transferring standard loopfuls to melted agar and pouring plates immediately and after 24, 48, 72 and 96 hours' incubation at 37° C.

The results of these experiments are tabulated below.

No. of tube	Controls		No. of tube	1:1,000			No. of tube	1:10,000			No. of tube	1:1,000			No. of tube	1:10,000		
	24 hours	48 hours		24 hrs	48 hrs	72 hrs		24 hours	48 hours	72 hours		24 hrs	48 hrs	72 hrs		24 hrs	48 hrs	72 hrs
1.	496,000	747,000	4 (a)	0	0	0	4 (b)	0	0	0	4 (c)	0	0	0	4 (d)	0	0	0
2.	424,000	596,000	5 (a)	0	0	0	5 (b)	0	0	0	5 (c)	0	0	0	5 (d)	0	0	0
3.	268,000	408,000	6 (a)	0	0	0	6 (b)	0	0	0	6 (c)	0	0	0	6 (d)	0	0	0
7.	6,416,000	10,608,000	10 (a)	0	0	0	10 (b)	60,000	1,576,000	5,680,000	10 (c)	0	0	0	10 (d)	0	0	0
8.	6,272,000	9,712,000	11 (a)	0	0	0	11 (b)	488	141	217	11 (c)	0	0	0	11 (d)	0	0	0
9.	5,582,000	7,232,000	12 (a)	0	0	0	12 (b)	3	1	0	12 (c)	0	0	0	12 (d)	0	0	0

It will be seen that in the control tubes, 1, 2 and 3, containing gelatin without meat extract the staphylococci multiplied to a moderate extent, and the multiplication was greatest in tube 1 containing the largest amount of gelatin. Homoflavine and quinone in concentrations of 1 : 1,000 and 1 : 10,000 completely inhibited growth in gelatin alone (4 *a*, *b*, *c*, *d*, 5 *a*, *b*, *c*, *d*, and 6 *a*, *b*, *c*, *d*). In all the control tubes containing gelatin and meat extract (7, 8 and 9) great multiplication occurred, the numbers found bearing a relation to the amount of gelatin present. While homoflavine in a concentration of 1 : 1,000 completely inhibited growth in these media [tubes 10 (*a*), 11 (*a*), 12 (*a*)] multiplication occurred at a concentration of 1 : 10,000 in the tube 10 (*b*) containing the most gelatin. In tube 11 (*b*) containing less gelatin the organisms diminished in numbers but some remained alive. Almost complete inhibition occurred in tube 12 (*b*) containing the least gelatin. Quinone in both concentrations inhibited growth in all cases.

These experiments like the last show that the action of quinone is much less affected than the action of homoflavine by the presence of gelatin.

*The influence of homoflavine and quinone on the liquefaction of gelatin by B. pyocyaneus.*

Media of the following composition were prepared and sterilised by boiling. After cooling the solution of the compound and one drop of an emulsion of *B. pyocyaneus* in distilled water was added.

	Meat extract	Gelatin (20%) in distilled water	N/10 soda	N/10 HCl	Water	Solution of compound
A.	1.0	2.0	0.56	—	0.94	0.5
B.	1.0	2.0	0.26	—	1.24	0.5
C.	1.0	2.0	0.1	—	1.4	0.5
D.	1.0	2.0	—	0.1	1.4	0.5

The cultures were incubated at 37° C. and cooled daily in a stream of water to see if liquefaction had taken place. Subcultures made on the second day showed numerous colonies in all the tubes, indicating that, under the conditions of these experiments, 1 : 1,000 concentrations of homoflavine and quinone did not completely inhibit the growth of *B. pyocyaneus*. The results are shown in the following table (p. 26).

It will be seen that the concentrations of homoflavine used, although they do not destroy the organisms, tend to inhibit the action or the production of the liquefying ferment most strongly when the medium is alkaline, while these concentrations of quinone act in the same manner most efficiently in series C, which is nearly neutral.

Other experiments of the same kind indicated clearly that the results were correlated with the number of organisms introduced. With a large initial dose liquefaction occurred in the 1 : 2,000 concentration of homoflavine on the fourth day in series A, and in the 1 : 1,000 concentration of quinone on the third day in series D.





Bashford, Hartley and Morrison (1917) in recording their experience of the action of acriflavine on wounds say that "The only favourable feature has been that the patient is apparently protected in some way from the absorption of toxic products." Possibly this protection is due to the influence of the dye on ferment action as illustrated in the experiment just quoted.

*Summary of experiments with homoflavine and quinone.*

	Homoflavine	Quinone
1 c.c. meat extract, 3.5 c.c. water, 0.5 c.c. solution of com- pound.	<i>B. coli</i> inhibited at concentration of 1 : 150,000, staphylococci of 1 : 300,000, <i>B. pyocyaneus</i> of 1 : 20,000.	<i>B. coli</i> inhibited at concentration of 1 : 90,000, staphylococci of 1 : 50,000, <i>B. pyocyaneus</i> of 1 : 7,500.
Alkaline meat ex- tract.	Efficiency greatly increased by small additions of N/10 soda.	Efficiency decreases as alkalinity increases. Most efficient at or near neutrality.
1 c.c. meat extract, 2 c.c. agar, 1.5 c.c. water, 0.5 c.c. solu- tion of compound.	<i>B. coli</i> inhibited at a concentra- tion of 1 : 4,500, staphylococci of 1 : 10,000, <i>B. pyocyaneus</i> of 1 : 500.	<i>B. coli</i> inhibited at a concentra- tion of 1 : 90,000, staphylo- cocci of 1 : 70,000, <i>B. pyocy- aneus</i> of 1 : 25,000.
Alkaline meat ex- tract agar.	Efficiency increases with alka- linity.	Efficiency decreases as alkalinity increases.
Growing cultures.	Much greater concentrations re- quired to inhibit growing cul- tures than organisms added to fresh media.	Action in this respect same as that of homoflavine.
	The oldest cultures are the most susceptible to prolonged ac- tion.	Action in this respect same as that of homoflavine.
Heating to 100° C.	Heating has little effect on its action.	Heating very greatly decreases the efficiency.
Initial dose of organ- isms.	Efficiency decreases in relation to the numbers of organisms added. With moderate num- bers there is first a diminution and then a multiplication.	Relation of efficiency to numbers less marked than with homo- flavine, and less tendency to multiplication after initial de- crease.
Gelatin in meat ex- tract.	Gelatin in the presence of strong concentrations of the dye re- tards the inhibitory action. In moderate concentrations mul- tiplication occurs if sufficient gelatin present.	Action little affected by the presence of gelatin.
Liquefaction of gela- tin by <i>B. pyocyaneus</i> .	Inhibited when medium alka- line.	Inhibited when medium neutral.
Egg albumin.	In strong concentrations of the dye retards the inhibitory action.	Action little affected.

## Crystal Violet.

As shown in Table I crystal violet has relatively little action on *B. coli* or *B. pyocyaneus*, and consequently the few additional experiments carried out with this dye were made with staphylococci.

Experiments made with media of the following composition show that this dye like homoflavine acts best when the medium is slightly alkaline.

	Meat extract	Agar	Composition of medium			Solution of dye	Concentration neces- sary to inhibit the growth of staphylococci
			N/10 soda	N/10 HCl	Water		
A.	1.0	2.0	—	0.15	1.35	0.5	1 : 1,500,000
B.	1.0	2.0	0.08	—	1.42	0.5	1 : 3,500,000
C.	1.0	2.0	0.58	—	0.92	0.5	1 : 5,000,000

In neutral meat extract the growth of staphylococci was found to be inhibited by a concentration of 1 : 10,000,000, but the precise limits were not worked out.

Crystal violet acts more efficiently on staphylococci growing in meat extract than on ox serum.

## Conclusions.

In cultures the effects of homoflavine and quinone, the two compounds most thoroughly investigated, vary on each species of organism with every change in the composition of the medium, whether the change is brought about by altering the proportion of any constituent or by the introduction of fresh constituents, and also with variations in the numbers and age of the organism. Again in each medium the concentration of the compound which inhibits each species of organism differs, and it is probable that yet other concentrations are required when mixed cultures are employed, though no experiments have been made to establish this point.

In wounds the conditions are more complex than in cultures. The conditions prevailing in no two wounds are likely to be identical, and in every wound the conditions are constantly altering, not only in regard to the chemical constituents of the fluids but also in regard to the numbers of organisms and the species and the relationships between them.

The work of Douglas, Fleming and Colebrook (1917) indicates "that bacterial symbiosis may play a very important rôle in wound infections." "Streptococci multiply much more rapidly when grown in symbiosis with various bacteria, amongst these being the group of diphtheroid bacilli which are present in practically every infected wound from the earliest stage until cicatrization is complete."

The conditions prevailing in a wound would be more closely simulated if frequent small additions of food substance were made to cultures. Under these conditions much greater concentrations of homoflavine would undoubtedly be required to cause complete inhibition of growth.

If any arguments based on results<sup>1</sup> in cultures of the type employed may be applied to wounds or other lesions associated with bacteria the following conclusions seem to be permissible in view of the experiments which have been quoted.

(1) No satisfactory results may be expected from the use of a dye or allied compound as a bactericidal agent unless the wound has been thoroughly cleansed before its application, since the complex organic fluids present are likely to interfere with the action of the solution.

(2) The most beneficial results are likely to be obtained if the solution of the compound is made in a fluid of the reaction at which the compound acts most efficiently, provided such a reaction is not in itself harmful to the tissues.

(3) Some compounds are more efficient than others against certain species of bacteria. In each case the dyes or other compounds used should possess special efficiency against the organisms ascertained to be present in the wound or lesion.

(4) Solutions of these compounds are most likely to produce satisfactory results if used in the very early stages of infection when the organisms are few, and unaccustomed to the new conditions in which they find themselves. Apart from killing the organisms or checking their growth the inhibitory action of certain of these compounds on some of the ferments would tend to render the conditions less favourable for bacterial growth and to hinder the production of toxic substances. When the organisms are very numerous, growing rapidly accustomed to their surroundings and protected in the fluids, these compounds are likely to be much less effective.

The last two conclusions based on culture experiments receive some support from clinical experience with acriflavine. Kellock and Harrison (1917) say that "an interesting point noticed lately has been that the antiseptic flavine appears to have no effect on *B. pyocyaneus*," and Taylor (1917) makes the following statement: "A large number of bacteriological examinations of wounds under treatment with different solutions has been recorded and instances of the specific action of certain dressing solutions demonstrated."

Drummond and McNee (1917) conclude that "flavine has many advantages as a *primary* treatment," but state that it is "not a success in the later stages." Pearson (1918) states that "in cases where infection and sepsis are active and uncontrolled the use of flavine following suitable operative measures has no beneficial effect on the subsequent progress of the case in so far as the control of sepsis is concerned."

<sup>1</sup> "It is generally recognized that the testing of substances for their antiseptic or germicidal properties is fraught with innumerable pitfalls and that by varying the conditions of testing almost any kind of result may be obtained" (Dakin and Dunham, 1917).

## APPENDIX

## CLINICAL OBSERVATIONS.

Several observers very kindly tested the action on wounds of a solution of 1 : 10,000 homoflavine and 1 : 100,000 crystal violet in distilled water. Many of these tests were carried out with the greatest care, the descriptions being accompanied by charts showing the previous treatment, the age and extent of the lesion, the rate of healing, the organisms found in smears and in cultures, etc. In some instances the progress of two similar wounds in the same individual, one treated with the solution mentioned and one with some other antiseptic, was compared. The results are described as "dramatic," "very successful," "successful," "moderate success" and "unsuccessful."

Careful though many of these inquiries have been they have not revealed the causes of the very divergent results, which occurred in the experience of several observers, and consequently it does not seem necessary to publish the cases in detail.

All the observations were made on relatively old wounds, and though they afford no evidence regarding the efficiency of the solution in the primary treatment of wounds, they show that old, infected wounds may be treated sometimes with "very successful" results. To ascertain the conditions under which successful results may be expected in such lesions requires combined bacteriological and chemical investigations on a series of suitable cases. Bacteriologically the species, number, rate of multiplication and symbiotic relationships of the organisms, and chemically the reactions of the fluids, the substances which inhibit the actions of the compounds, and especially the products of tissue decomposition, due to bacterial or tissue ferments, available as food material for the various species of bacteria, influence the results.

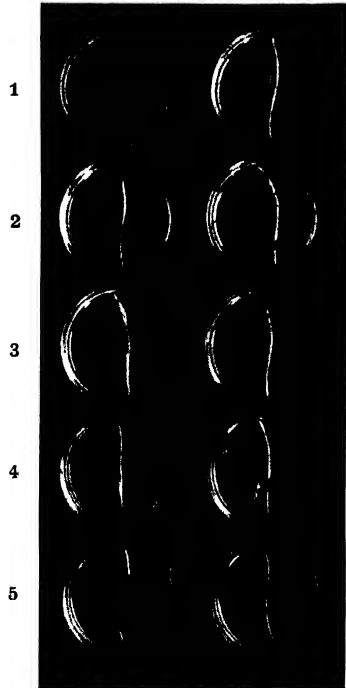
Apart from wounds this solution was employed with varying results in the treatment of gonorrhoea. Satisfactory and even "dramatic" results were obtained in several cases of stomatitis, gingivitis and pyorrhoea.

A 1 : 10,000 solution of quinone in distilled water has been tested on a small number of wounds with varying results. Like homoflavine it has given excellent results in some cases of pyorrhoea.

The three compounds differ so greatly in their powers of inhibiting the growth of such organisms as *B. coli*, staphylococci and *B. pyocyaneus* under varying cultural conditions that it might be desirable to test the action on wounds of a solution in distilled water of 1 : 10,000 homoflavine, 1 : 10,000 quinone and 1 : 100,000 crystal violet.

Pharmacologically these compounds are very inert, as shown by experiments which Professor A. R. Cushny, F.R.S., Dr W. E. Dixon, F.R.S., and Dr D. Cow very kindly carried out.

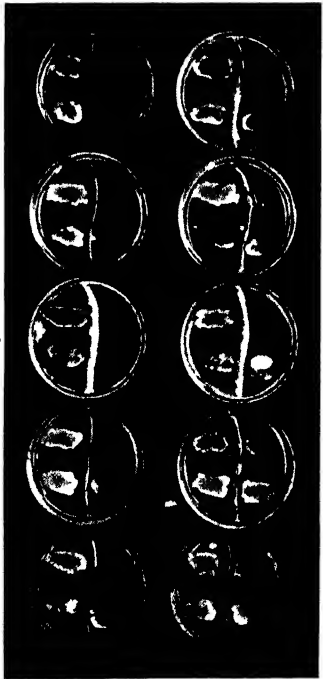




Figs. 1—10

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Figs. 11—20

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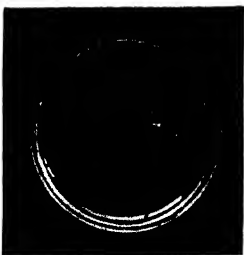


Fig. 21

## EXPLANATION OF PLATE.

Figs. 1-20. Photographs to show the differences of growth on agar after 24 hours' and after 48 hours' incubation at 37° C.

Figs. 1-10 represent Petri dishes divided according to Churchman's (1912) method after 24 hours' incubation at 37° C. The left side in each case is the control, and contains a medium composed of agar 2 c.c., ox pancreas extract<sup>1</sup> 0.5 c.c., distilled water 1.0 c.c., and N/10 soda 0.5 c.c. The right side contains a medium composed of agar 2 c.c., ox pancreas extract 0.5 c.c., distilled water 0.6 c.c., N/10 soda 0.5 c.c., and the solution of homoflavine 0.4 c.c. The concentration of homoflavine in dish 1 was 1 : 100,000, in dish 2 1 : 200,000 and so on up to 1 : 1,000,000 in dish 10.

An emulsion of *B. coli* in distilled water was streaked across the upper part of each side of each dish and an emulsion of staphylococcus across the lower part of each side.

Figs. 1-10 show the growth of *B. coli* in each case on the control side, but in most cases no visible growth of the staphylococcus. On the right side no visible growth of either organism has occurred in 24 hours at 37° C.

Figs. 11-20 represent the same dishes after 48 hours' incubation. On the left or control sides considerable growths of both *B. coli* and staphylococcus are seen. On the right sides a slight growth of staphylococcus is seen first in fig. 14 with a concentration of 1 : 400,000 homoflavine, and it becomes more marked in the later dishes, being almost equal to the controls for fig. 16 (homoflavine 1 : 600,000) onwards. Growth of *B. coli* is seen first in fig. 16 with a concentration of 1 : 600,000 homoflavine. In concentration of 1 : 900,000 and 1 : 1,000,000 (figs. 19, 20) the growths are equal to the controls.

These photographs indicate sufficiently the necessity for adopting a rigid time limit in recording the results of such experiments.

Fig. 21. A Petri dish on the bottom of which two vertical streaks of plain agar were made (seen as light coloured vertical bands), and allowed to set. Over them was poured a medium composed of meat extract 1 c.c., agar 2 c.c., N/10 soda 0.08 c.c., distilled water 1.42 c.c. and 0.5 c.c. of a solution of homoflavine, making a final concentration of 1 : 17,000.

An emulsion of *B. coli* was streaked transversely across the upper part of the dish, and the colonies of this organism are seen stretching across the dish. Across the lower part of the dish an emulsion of staphylococcus was streaked. It will be noticed that the colonies of this organism are growing only over the streaks of plain agar.

The photograph represents the condition after 48 hours' incubation at 37° C.

<sup>1</sup> Numerous experiments with pancreas and other organ extracts were made, but have not been quoted, as new factors are introduced which have not been worked out sufficiently.

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# THE TESTING OF ANTISEPTICS IN RELATION TO THEIR USE IN WOUND TREATMENT<sup>1</sup>.

BY C. H. BROWNING AND R. GULBRANSEN.

(From the Bland-Sutton Institute of Pathology, the Middlesex Hospital.)

IN previous reports (Browning, Gulbransen, Kennaway and Thornton) attention was directed to the value of certain experimental tests of antiseptics as an indication of their practical value in the treatment of infected wounds, namely the estimation of antiseptic potency in the presence of serum and the relationship between this property and toxicity to tissues. In regard to the latter factor it was pointed out that toxicity must be investigated on a wide basis; thus determinations were made of the effect of antiseptics on leucocytes, as shown by alterations in their phagocytic properties, and on an epithelial membrane, *e.g.* the conjunctiva, as evidenced by the production of inflammation, and also the toxicity to the body as a whole was estimated in cases where the substance was readily absorbed (see Table I). It was emphasised,

Table I.

Substance	Maximum non-lethal dose for 20 gram mouse	Bactericidal concentration for			
		Staph. aureus in		B. coli (Escherich) in	
		0.7% peptone water	Serum	0.7% peptone water	Serum
Diamino-acridine sulphate (proflavine)*	0.003 gram	1 : 20,000	1 : 200,000	1 : 4,000	1 : 100,000
Diamino-methylacridinium chloride (acriflavine)*	0.0006 „	1 : 20,000	1 : 200,000	1 : 1,300	1 : 100,000
Phenol	0.006 „	1 : 250	1 : 250	1 : 500	1 : 500
Mercury perchloride	0.0001 „	1 : 1,000,000	1 : 10,000	1 : 1,000,000	1 : 10,000

*Method of the Tests.* The toxicity for mice was determined by injecting watery solutions subcutaneously, the dose being so arranged that 20 grm. mouse received a volume of 1 c.c.: to animals of other weights corresponding volumes were given, but mice not exceeding the limits of 15–25 grm. were selected for the tests.

\* It has been suggested that the increased bactericidal action of the flavines in serum is merely an instance of a general enhancement of toxicity due to serum; as might be anticipated the injection of the dose in 80 per cent. serum instead of a watery solution does not, however, alter the fatal dose.

however, that the susceptibility of different tissues toward a given chemical compound may vary greatly and that this important character in substances destined for use as antiseptics requires much further investigation; the impossibility of using strychnine, even if it were a potent antiseptic, owing

<sup>1</sup> A Report to the Medical Research Committee.

to its specialised toxic action on the nervous system was cited. The result of our work on these lines pointed to the value of certain basic benzol derivatives—*brilliant green*—and acridine compounds—*acriflavine* and *proflavine*. Independent clinical observations (Ligat, James) supported the view that the sum total of tests which we had applied, constituted a trustworthy guide to the therapeutic value of these antiseptics and afforded indications as to the suitable means of application. In the interest of further progress it was obviously of great importance that the significance of suitable laboratory tests should be established as a guide to practical use, since such investigations constitute an essential preliminary to clinical trials. Subsequent observations by Drummond and McNee, Carslaw, Carslaw and Templeton, and Pilcher and Hull, have added greatly to our knowledge of the therapeutic value of the flavine compounds both for prevention and treatment of sepsis, and the clinical material on which the reports are based (over 5000 cases in Pilcher and Hull's series) indicates the representative nature of the tests. From these investigations the result emerges that, employed in conjunction with operative measures, salts of certain basic organic compounds, especially the flavines and also brilliant green, are capable of yielding valuable therapeutic results when applied by the relatively simple and rapid method of packing with gauze soaked in—not wrung out of—a solution of the antiseptic and an important feature of the flavines is that infrequent renewal in the wound suffices to maintain their effect. This may be advantageously provided for, on occasion, by a valved tube according to Kellock's method. The satisfactory action of infrequent renewal of the antiseptic seems clearly to be correlated with the continued activity of the flavine compounds in the presence of serum. The importance of interrupting the application of flavine after a certain stage, owing to an inhibition of healing—not to any destructive action on existing tissues—which may supervene, is a valuable practical contribution to our knowledge of the use of these compounds which has been established by the above-named clinical observers (see also Bashford, Hartley and Morrison). Several investigators, however, have published results of laboratory investigations tending to the conclusion that we have exaggerated the properties of the flavine compounds; accordingly, a number of points which have been raised relative to the action of these antiseptics on bacteria will be dealt with here. Their action on the tissues will be discussed elsewhere.

#### THE CHOICE OF A MEDIUM FOR TESTING ANTISEPTICS.

*Serum* was originally selected because serous exudate constitutes practically the diluent to which an antiseptic is exposed in a wound, both when recent and, also at a later stage when a granulating surface is mechanically cleansed from pus. The fact that serum intensifies the bactericidal action of the flavines, especially in the case of *B. coli*, has been met by the statement that serum is a poor culture medium and it has been suggested that the intensifying effect is merely a summation of the inhibitory properties of serum

and of the antiseptic. The error of these views becomes clearly apparent when one tests a number of specimens of serum and also a variety of antiseptics. Thus, while some specimens of ox serum afford a relatively poor medium for the proliferation of *B. coli*, others yield excellent growths. On what this depends has not been determined; the unsuitable sera are usually those which are practically colourless; but the difference has not been found to depend on factors such as length of contact with the clot or the addition of a trace of laked red corpuscles. The intensifying action of serum on the bactericidal property of flavine is well shown in the case of sera which are favourable to bacterial growth. The addition to the serum of an amount of trypsin (see Douglas and Colebrook) which augments the properties as a medium of unfavourable specimens, has not been found to alter distinctly its intensifying action for flavine<sup>1</sup>. The summation theory is further disproved by finding substances which are extremely potent antiseptics in watery medium, but whose action is greatly reduced by serum, *e.g.* in the case of brilliant green with *B. coli*—

Concentration in 0.7 % peptone water which kills = 1 : 130,000.

Concentration in serum which fails to kill = 1 : 5000 (result obtained by subculture after 48 hours: the control culture in serum without antiseptic yielded abundant growth).

If admixture with serum, in addition to merely diluting the antiseptic, also neutralises its effects, then frequent renewal of the substance will be essential in order to obtain efficient action in a wound. This is the case with all the common older antiseptics in watery solution except carbolic acid; such frequent renewal exposes the body as a whole to the danger of toxic effects should absorption occur; phenol and mercuric chloride are potent poisons when absorbed, hence their applicability is strictly limited. On the other hand, the hypochlorites have the great advantage of being converted into harmless compounds in contact with the tissues, but the "complicated hydraulic system," as Dakin, Lee, Sweet, Hendrix and Le Conte term it, which is necessitated by the unstable nature of these antiseptics in watery solution, in order to obtain efficient action, renders the suggestion of their general use practically a counsel of perfection. Brilliant green (first used by Leitch, see also Ligat and Webb) although diminished in action by serum, is highly potent and is scarcely absorbed; on the other hand, the flavines while absorbed are very little toxic, as was demonstrated by the fact that relatively large amounts could be administered intravenously (intravenous administration has now been practised on an extensive scale in cases of trench fever—Byam, Dimond, Sorapure, Wilson and Peacock).

As regards the action of other media, *pus* diminishes the antiseptic power

<sup>1</sup> Subsequent experiments have shown that the commercial preparation of trypsin which has been recommended for use varies considerably, thus two specimens acted as above stated; a third, however, in similar concentrations produced great alteration of the serum, as shown by abundant formation of tyrosine crystals without any bacterial contamination. Such profoundly altered serum ceased to give a marked intensification of the antiseptic effect of the flavines. This point is being investigated further.

of the flavines, as was shown in our first report (confirmed by Fleming, and Parry Morgan). *Defibrinated blood* diminishes the action as we demonstrated (confirmed by Fleming, Dakin and Dunham, Parry Morgan).

*Milk* diminishes the action (Hewlett), so does *minced meat* (Fleming), which also removes the bactericidal properties of blood serum. Dakin and Dunham have used further a mixture of watery muscle extract with serum. It would, of course, be of great value if an antiseptic were to act equally well in all media, but, this is probably impossible and even if an antiseptic could be disseminated in potent concentration by the blood stream it would still fail to penetrate effectively into any considerable mass of necrotic tissue, hence operative interference must be an essential factor in wound treatment. As regards the use of these other media, whose effect in neutralising flavine seems to have been regarded as an important observation by some of the investigators above mentioned, no cogent argument has been adduced to show that, as compared with serum, all or any of them constitute a more rational test medium for ascertaining the value of antiseptics in wounds. Milk and minced meat are clearly of only remote application, while, as we have repeatedly pointed out, pus is not the medium in which an antiseptic is required to act. Before application of an antiseptic pus and necrotic tissue should be removed mechanically; the layer of granulation tissue into contact with which the solution then comes is widely different from pus in its physical characters. We have throughout endeavoured to employ tests which possess "heuristic" value, and consider that the test in serum has been shown to belong to this category, whereas no proof has been afforded that the others do. It has been pointed out that the flavines are compatible with hypertonic saline up to 5 % NaCl; hence a means was provided for regulating to some extent the amount of serous fluid in the wound. This combination of flavine with hypertonic saline has been advantageously employed by Pilcher and Hull.

#### METHODS OF TESTING ANTISEPTIC POTENCY—BACTERIOSTATIC ACTION IN RELATION TO TOXICITY (EFFECT ON PHAGOCYTOSIS).

We pointed out originally that the flavines and brilliant green bring about the death of bacteria comparatively slowly; but apart from lethal action, very great dilutions of these compounds are effective in checking bacterial multiplication, that is, they exhibit a high degree of *bacteriostatic* action, to use Gildersleeve's term, and so act as potent antiseptics *sensu stricto*. On the other hand, substances such as mercuric chloride, phenol and chloramine-T all produce their maximum effect rapidly (within two hours) and no significant action occurs subsequently, *i.e.* concentrations of the latter substances which fail to prove lethal quickly, exert little or no effect on the multiplication of surviving bacteria. In addition, when it is remembered that serum neutralises these antiseptics—except phenol—the need for frequent renewal becomes clear; but the high toxicity of mercuric chloride and of phenol (which is a

very weak antiseptic) in relation to their antiseptic potency preclude unrestricted renewal.

It appeared that bacteriostatic action was a most valuable property for wound therapy and, because the serous exudate in the wound did not neutralise the flavines, mere slowness of lethal action as determined *in vitro* mattered little; the organisms under the influence of the antiseptic were, so to speak, controlled from the beginning. A striking confirmation of this is afforded by the observations of Drummond and McNee and also of Carslaw and Templeton, who found that in spite of the presence of bacteria in wounds treated with flavine there is a notable absence of both the local and the general phenomena of inflammation and of septic infections. Experimental evidence bearing on the co-operation of antiseptics with the tissues in overcoming infection will be published elsewhere.

Methods of testing antiseptics *in vitro*, whose sole aim is to detect whether or not every viable organism has been destroyed, may fail altogether to afford a true estimate of the potency of a substance for therapeutic purposes. Thus, Fleming and Hewlett inoculated fluid medium with the mixture of organisms and antiseptic and so obtained no information as to the numbers of organisms which had been killed short of total sterilisation. We have always made subcultures from the antiseptic mixtures on to solid media, so as to determine, by means of the number of colonies, whether the bacteria had increased or decreased. As the inoculation dose of organisms we originally chose a relatively minute number; the medium is not thereby rendered turbid at the time of inoculation, accordingly, if the mixture develops opacity later on, this indicates that free proliferation of the organisms has occurred. It was felt, however, that the use of a minute inoculation exposed our work to the objection that the antiseptic could not inhibit larger numbers of bacteria; accordingly, our second report contained experiments with much larger amounts of organisms, and it was shown in the case of acriflavine and *B. coli* that a twenty-thousand-fold increase in the inoculation dose necessitated only about a two-and-a-half-fold increase in the amount of antiseptic required to produce complete sterilisation. However, without direct reference to this latter work, our results with the basic organic antiseptics have been ascribed by Fleming, Hewlett, and Dakin and Dunham to the use of minute doses of organisms, which have been stated to lead to an exaggeration of the potency of the flavines as compared with other compounds. Accordingly, further tests have been performed, using larger amounts of organisms in conformity with the experiments of Dakin and Dunham.

The following is an example:

A 24-hour agar slope culture of *Staphylococcus aureus* was suspended in 5 c.c. of 0.85 per cent. NaCl solution: 0.025 c.c. each of a 1 : 100,000 and 1 : 1,000,000 dilution of this suspension when plated on agar yielded respectively 175 and 21 colonies, hence it may be concluded that the stock suspension contained about 750 million organisms per cubic centimetre.

To a series of tubes each containing 1 c.c. ox serum (heated previously for  $\frac{1}{2}$  hour at 55° C.) 0.1 c.c. of the *Staphylococcus* suspension was added and also 0.1 c.c. of varying dilutions of the antiseptic in water ("strong inoculation" series). In the "weak inoculation" series the inoculation dose of staphylococci was 0.1 c.c. of a 1 : 1000 dilution of a 24-hour peptone water culture. After incubation of the mixtures of organisms and antiseptic at 37° C. for 24 hours a loopful from each tube was stroked on agar; which was then incubated for 48 hours. The results were as follows:

Table II.

*Results of Subculture from Mixtures of Staphylococcus aureus and Flavines after 24 hours' contact at 37° C.*

Concentration of antiseptic	ACRIFLAVINE		PROFLAVINE	
	Weak inoculation	Strong inoculation	Weak inoculation	Strong inoculation
1 : 400,000	Marked diminution	—	8 colonies	—
1 : 200,000	Sterile	—	Sterile	—
1 : 130,000	"	Diminution	"	Diminution
1 : 100,000	"	"	"	"
1 : 40,000	"	Sterile	"	About a dozen colonies
1 : 20,000	"	"	"	" " "
1 : 13,000	"	"	"	" " "
				(Sterile in 48 hours)
1 : 4,000	"	"	"	Sterile

Controls after incubation:

*Weak* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 22 colonies

*Strong* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 36 colonies.

Parallel experiments with the *strong* inoculation in a medium containing 33 per cent. serum yielded the same result as those with full serum.

In this and the following table "Diminution" = diminished number of colonies in subculture as compared with control without antiseptic.

In all cases the culture tubes were well shaken before making subcultures, so that any fallacy due to spontaneous sedimentation is excluded.

It might be objected that a loopful was an insufficient sample to take from each specimen; but the fact that a loopful of a 1 : 100,000 dilution of the inoculated serum controls without antiseptic, after incubation, yielded several dozen (22-36) colonies (see also Table III), proves that there is here no fallacy. Since the large inoculation dose, as employed by Dakin and Dunham, imparts a marked turbidity to the mixture, it was considered important to determine the relative number of viable organisms present in the control tubes containing serum, but no antiseptic, not only at the commencement of the experiment, but also after incubation. In order to do this a series of decimal dilutions was prepared in both cases and a loopful from each was stroked on agar; this simple procedure may be recommended as of sufficient accuracy for practical purposes, while it saves time and materials consumed by plating a series of dilutions. The results after incubation of the plates for 48 hours were as follows:

Table III.

Dilution	Serum <i>plus</i> Weak inoculation subcultured		Serum <i>plus</i> Strong inoculation subcultured	
	at once	after 24 hours at 37° C.	at once	after 24 hours at 37° C.
Undiluted	20 colonies	Homogeneous stroke	Homogeneous stroke	Homogeneous stroke
1:10	—	Slight decrease in density of growth	Homogeneous stroke	Discrete but closely adjacent colonies
1:100	—	Discrete but closely adjacent colonies	Slight decrease in density of growth	„
1:1,000	—	Marked decrease in number of colonies	Discrete but closely adjacent colonies	„
1:10,000	—	55 colonies	„	Marked decrease in number of colonies
1:100,000	—	22 „	„	36 colonies

The results illustrated in Tables II and III show:

(1) Controls: the large amount of organisms, as used for inoculation by Dakin and Dunham, did not maintain itself, *i.e.* in spite of some proliferation, as evidenced by increase in turbidity of the culture, the large inoculation led to increased death of bacteria, so that viable organisms were more numerous at the beginning than at the end of the experiment. It is obvious, therefore, that this is not the most suitable amount of organisms for testing the properties of a progressively acting antiseptic, since under the conditions arranged by Dakin and Dunham the organisms diminish in the absence of any antiseptic.

(2) With *Acriflavine*, in spite of the enormous number of organisms in the strong inoculation series, the lethal concentration (1:40,000) was not more than five times that found with the weak inoculation; further, a concentration of 1:130,000 produced a definite lethal effect on the organisms in the strong inoculation series. In the case of *Proflavine* it is clear that the result for practical purposes is the same, but there is a longer range of persistence of viable organisms before complete sterility is attained. Thus a loopful from the *undiluted* mixture containing proflavine 1:40,000 yielded only a dozen colonies, whereas the control without antiseptic when diluted 1:100,000 yielded 36 colonies in a loopful; therefore there can be no question as to the powerful bactericidal action exerted by this high dilution of the antiseptic. Such an effect will fail to be observed when testing results merely by subculturing into fluid medium, as practised by Fleming and Hewlett, where one viable organism will yield a growth; this would account for their conclusion that our original findings were exaggerated.

The following is a similar experiment in which acriflavine and proflavine were tested with large and small amounts of *B. coli*.

Method as above: the strong inoculation contained about 5000 million more organisms than the weak inoculation. The results are shown in Table IV

The Control *weak* inoculation subcultured at once yielded 220 colonies in a loopful and after incubation 1:10,000, 1:100,000, and 1:1,000,000 dilutions yielded 65, 30 and 2 colonies in a loopful respectively.



Table IV.

*Results of Subculture from Mixtures of B. coli and Flavines after  
24 hours' contact at 37° C.*

Dilution	ACRIFLAVINE		PROFLAVINE	
	Weak inoculation	Strong inoculation	Weak inoculation	Strong inoculation
1 : 400,000	Growth	—	Diminution	—
1 : 200,000	A few colonies (sterile in 48 hours)	—	A few colonies (sterile in 48 hours)	—
1 : 100,000	do.	Growth	Sterile	A few colonies (sterile in 48 hours)
1 : 40,000	—	A few colonies (sterile in 48 hours)	..	Sterile
1 : 20,000	—	..	..	..
1 : 13,000	—	Sterile	..	..

Controls after incubation:

*Weak* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 30 colonies.

*Strong* inoculation without antiseptic—one loopful of a 1 : 100,000 dilution yielded 1 colony.

Parallel experiments with 33 per cent. serum medium gave the same result as full serum in the case of the strong inoculation.

The Control receiving the *strong* inoculation yielded 118 colonies in a loopful of a ten-thousand-million-fold dilution at once, but after incubation for 24 hours a loopful of 1 : 10,000, 1 : 100,000 and 1 : 1,000,000 dilutions yielded 30, 1 and 0 colonies respectively; hence with *B. coli* the strong inoculation shows even a more marked disadvantage as a test method than in the case of staphylococcus, owing to spontaneous death of the organisms.

It is striking that in the case of *B. coli* as compared with *Staphylococcus aureus* the behaviour of acriflavine and proflavine seems to be reversed and the former now tends to cause the wider zone of bacteriostatic action before complete sterilisation is attained; whether this is the invariable result with the two substances we have not sufficient evidence to show, but the general result is the same with both compounds, viz. that the strong inoculation requires for practically complete sterilisation at the most  $2\frac{1}{2}$  times the minimum lethal concentration required by the weak inoculation.

Accordingly, great increase—many million-fold—in the number of organisms in the inoculation dose, causes merely an insignificant increase— $2\frac{1}{2}$ –5-fold—in the concentration of the flavines necessary for practically complete sterilisation with *Staphylococcus* and *B. coli*; further, the occurrence of bacteriostatic action in much higher dilutions is a characteristic property of these substances.

We would again specially emphasise the therapeutic value of substances, like the flavines, which possess such powerful bacteriostatic properties and which are at the same time relatively non-toxic to the tissues and are not neutralised by serum. We had previously drawn attention to the ratio

concentration of substance which inhibits phagocytosis  
concentration causing death of organisms in serum

as one means of measuring the suitability of antiseptics for acting in a wound. The significance which we attached to this therapeutic coefficient has been

questioned because the time of exposure of organisms and of leucocytes to the antiseptic was not the same (Fleming), but this criticism fails altogether to take account of the fact that, short of producing actual death of the organisms, bacteriostatic action is exerted by these antiseptics. Thus, although the bacteria may still be capable of proliferating when removed into culture medium and out of contact with the antiseptic, this is of little practical importance as compared with the fact that their activities are inhibited when the antiseptic is present. Wright has also recently emphasised as an "all important principle" that the actual state of bacteria under conditions unfavourable to their activity (as where they are in contact with leucocytes in his observations or with antiseptics in our work) is not accurately shown by a method which proceeds to place the organisms under the most favourable circumstances for producing a culture.

In the case of the flavine antiseptics there exists such a wide range between the weakest concentration which will suffice to inhibit and eventually to kill organisms in a serous medium on the one hand, and on the other, the great strength which is required to paralyse phagocytosis<sup>1</sup> (see our previous reports), that antiseptic action and the natural defensive processes can be expected to go on side by side. No other antiseptic which has been tested, affords results which justify the belief that with it such will occur to anything approaching the same extent.

Of late there has been a tendency to question the importance of the leucocytes as a defensive mechanism in infections. Thus, prominence has been given to the fact that organisms which have been ingested by leucocytes, may be protected from the lethal action of antiseptic solutions (Jones and Rous); further, dissemination of infection has been attributed to the transport of organisms enclosed in leucocytes, and recrudescence has been explained by renewed activity of phagocytosed bacteria. The recent important experiments of Alexander, however, serve to restore perspective to the view of the anti-infective properties of the leucocytes—this worker found that virulent pneumococci when incubated along with antipneumococcus serum and leucocytes for a few hours (six), became attenuated, although they were not killed, whereas neither the antiserum nor the leucocytes by themselves produced this effect on the organisms. Thus, there is a good reason for taking into consideration the action of any proposed therapeutic agent on the activity of leucocytes when considering the properties which will determine its efficacy in the treatment of infections.

<sup>1</sup> Parry Morgan has also investigated the occurrence of phagocytosis *in vitro* in the presence of acriflavine and, since marked agglutination occurred in the mixture, has expressed the opinion that possibly "the phagocytosis was more apparent than real and that the organisms merely adhered to the phagocytes and were not ingested." We did not consider it necessary to refer to this point in our first report, since we had found that similar agglutination occurred with other substances, but that owing to the toxic effect of the latter phagocytosis did not take place and there were then no appearances which resembled the ingestion of organisms by the leucocytes observed in the experiments with flavine; thus it was evident that *phagocytosis occurred in the presence of flavine*.

## THE TESTING OF ANTISEPTICS OF THE CHLORINE GROUP.

Dakin and Dunham have recently observed that "in making tests of the germicidal efficiency of any antiseptic there seems to be no good reason for not following the order of mixing the materials indicated by the conditions of practical use—namely, to add the antiseptic last to the inoculated medium." With reference to our work they proceed further, "the lethal concentration of chloramine-T in serum is stated to be 1 : 250 without reference to much lower concentrations already published by others. The discrepancy is due, in the main, to the fact that the antiseptic was added first to the medium and the organisms last." We have now repeated our experiments (*a*) in the manner postulated by Dakin and Dunham, and (*b*) in the way in which they were previously performed, *i.e.* adding the antiseptic to the serum and then adding the organisms within 2 to 3 minutes afterwards, *i.e.* following Dakin's own previous publication in which this statement appears, "*Determination of Germicidal Action*... A series of tubes each containing 5 c.c. of a solution of the substance at a progressively decreasing concentration is first of all prepared, and to each tube the organism is added... The tests carried out in the presence of blood serum were performed in the same way, only the liquid in the first series of tubes contained 50 per cent. of horse serum previously heated at 55°–56° C." The results in both series are identical and confirm our previous figure (see Table V). We have been able to show that the difference between our results and Dakin's is due mainly to the fact that Dakin originally employed 50 per cent. serum—later Dakin and Dunham used 33 per cent. serum—whereas in our experiments the antiseptic mixtures contained over 80 per cent. of serum (in our experience 80 per cent. heated ox serum is a more favourable medium for the growth of staphylococci than is 40–50 per cent. serum).

The following is an example:

Medium = ox serum—previously heated at 55° C. for  $\frac{1}{2}$  hour—in each tube 1 c.c., (*a*) of undiluted serum, (*b*) of serum diluted with an equal volume of 0.85 per cent. NaCl solution. Chloramine-T stock solution = 1 : 23.3 (the strength of this solution was verified by titration both immediately before and after the time of employment in the experiment). Inoculation dose of organisms = 0.1 c.c. of 1 : 1000 dilution of a 24-hour peptone water culture of *Staphylococcus aureus*. In series A the organisms were added to the medium first and then the chloramine solution; in series B the chloramine was added first and then the cocci after an interval of 2–3 minutes. As a control acriflavine in undiluted ox serum was tested. Sub-cultures were made on agar after 24 hours' incubation at 37° C. The results are shown in Table V.

These results confirm our original figures, although in the present instance 20 times as many organisms were employed for the inoculation as previously. It may be noted here that to dismiss the use of small numbers of bacteria as unsuitable for testing the action of an antiseptic *in vitro*, since a similar number in a wound borders on "surgical sterility," is entirely beside the point.

Table V.

*Action of Chloramine-T on Staphylococcus aureus.*

Concentration of chloramine	A		B	
	50 per cent serum	Full serum	50 per cent serum	Full serum
1 : 700	+	+	+	+
1 : 466	-	+	-	+
1 : 325	-	+	-	+
1 : 233	-	-	-	-

Control: concentration of acriflavine in undiluted serum, 1 : 400,000 +.

1 : 200,000 -.

+ = growth in subculture on agar.

- = no growth in subculture.

In a wound one is dealing with mechanisms inimical to the organisms (due to the leucocytes, etc.), which do not operate *in vitro*, hence the small number of organisms introduced into the culture medium is by no means subjected to the unfavourable conditions which may prevail in a wound.

## VARIATIONS IN RESISTANCE OF ORGANISMS TO FLAVINES

Acriflavine and proflavine are the most powerful antiseptics so far investigated for staphylococci and the ordinary types of *B. coli* in a serum medium. Certain strains of streptococci appear to be even more susceptible (Parry Morgan), which should be advantageous in view of the rôle of streptococci in infected wounds, although we did not find the "enterococcus" to possess more than average susceptibility. The existence of marked differences in the resistance of various species of organisms toward a particular antiseptic is now well known (see Browning). Drummond and McNee isolated from certain wounds organisms of coliform type which showed a high degree of resistance toward the flavines; we have had the opportunity of investigating three such strains and find that they all belong to a most unusual type of *B. coli*, which fails to form indol, also they are late lactose-fermenters: their culture reactions were as follows:

Motility +	$\left. \begin{array}{l} \text{glucose} \\ \text{lactose} \\ \text{saccharose} \\ \text{mannitol} \\ \text{maltose} \end{array} \right\}$	acid and gas	$\left. \begin{array}{l} \text{dulcitol} \\ \text{inositol} \end{array} \right\}$	no change	$\left. \begin{array}{l} \text{milk acid and late clot; indol} \\ \text{negative (12 days); gela-} \\ \text{tine not liquefied} \end{array} \right\}$

We have tested the action in serum of both acriflavine and proflavine on eleven types of *B. coli* and also on *B. pyocyaneus*, and *Urobacillus septicus*;

the resistant strains being also included in the series; the results were as follows:

<i>B. coli</i> (Escherich)	}	No visible growth in serum <i>plus</i> acriflavine or proflavine 1 : 200,000 after 48 hours at 37° C.
B. No. 71		
<i>B. Schafferi</i>		
<i>B. Grünthal</i>		
<i>B. neapolitanus</i>		
<i>B. vesiculosus</i> (2 strains)		
<i>B. lactis aerogenes</i> (2 strains)		
<i>B. Morgan I</i>		
<i>B. paracoli</i> type		
<i>B. coscoroba</i>		
B. No. 67 (inositol fermenter)	}	
<i>Urobacillus septicus</i> ( <i>proteus</i> class)		

Resistant (non-indol forming) coliform B. (3 strains)—visible growth in serum *plus* antiseptic 1 : 40,000; none in 1 : 20,000.

*B. pyocyaneus* (2 strains): visible growth in serum *plus* antiseptic 1 : 20,000; none in 1 : 10,000.

Inoculation dose in each case 0.1 c.c. of 1 : 1000 dilution of a 24-hour peptone water culture to 1 c.c. medium.

Controls without antiseptic all yielded abundant growth, with marked turbidity of the medium.

Parry Morgan, and Bashford, Hartley and Morrison also mention resistant coliform bacilli, but give no details as to their characters. It would, therefore, be quite misleading to suggest that the typical colon bacilli of faecal origin present such resistance to flavine antiseptics as to invalidate their use generally in wounds containing coliform organisms. *B. pyocyaneus* evidently belongs to the types most resistant to flavine, which has also been observed clinically (Kellock and Harrison), but this organism appears to be relatively unimportant as a pathogenic agent in wounds. It is interesting that *B. pyocyaneus* has also been found resistant to hypochlorites (Taylor).

#### SUMMARY AND CONCLUSIONS.

(1) *The antiseptic and bactericidal properties of Flavines and Brilliant Green.* Extended investigations have confirmed the original values. It has been shown that the inferior potencies recorded by certain other workers depend on the use of methods unsuited for the observation of antiseptic properties, *i.e.* they fail to detect inhibition of bacterial activity *i.e.* bacteriostatic action, which is exhibited to a marked degree by flavine and brilliant green.

(2) For the therapy of a local bacterial infection, as in a wound, such bacteriostatic action is of great value. It is not essential that the chemical

agent should by itself actually kill the organisms. Highly successful results can be obtained by a co-operation of the antiseptic and the tissues, so that the pathogenic action of the organisms is restrained. The flavines in virtue of their low toxicity to mammalian tissues and their high bacteriostatic power are therefore specially suited to act as local therapeutic agents. In addition, the fact that they are not neutralised by admixture with serum enables them to be applied clinically by a relatively simple method which does not necessitate frequent renewal.

(3) The "fundamental error" to which the method of testing chlorine antiseptics originally practised by Dakin, is liable, and which Dakin and Dunham have drawn attention to, has been shown not to affect our previous results with Chloramine-T. The difference between our values and the others is due to the fact that we employed 80 per cent. serum in the test medium, which is much more active in neutralising this antiseptic than is 33 to 50 per cent. serum employed by Dakin and his co-workers.

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# RECENT ADVANCES IN THE DIFFERENTIATION OF LACTOSE-FERMENTING (GAS-PRODUCING) BACILLI, WITH SPECIAL REFERENCE TO THE EXAMINATION OF WATER AND FOOD PRODUCTS.

By DENYS R. WOOD, F.I.C.,

*Public Analyst and Bacteriologist, Somerset County Council.*

*(From the Somerset County Public Health Laboratory.)*

IN recent years a considerable amount of work has been done by American bacteriologists on the differentiation of lactose-fermenting (gas-producing) organisms.

Rogers, Clark and Davis (1914) showed that by refined technique these organisms could be sharply differentiated into two main types by the estimation of the ratio of carbon dioxide to hydrogen produced in glucose media: those producing gases in the ratio  $\text{CO}_2 : \text{H}_2 =$  about 1.06 and characterised by the large proportion of strains producing indol, and those producing gases in the ratio  $\text{CO}_2 : \text{H}_2 =$  over 1.5 and characterised by the low proportion of strains producing indol.

Subsequently Rogers, Clark and Evans (1914) showed that practically only those organisms producing gases in the low ratio ( $\text{CO}_2 : \text{H}_2$ ) were to be found in bovine faeces, while in milk about 50 % belong to the low and 50 % to the high ratio ( $\text{CO}_2 : \text{H}_2$ ) type, and drew the inference that source of the latter must be sought elsewhere than in bovine faeces.

Later Rogers, Clark and Evans (1915) examined 166 cultures of glucose fermenters derived from various kinds of grain, 160 of these fermented lactose also, and 95 % of the lactose-fermenters were found to belong to the high ratio ( $\text{CO}_2 : \text{H}_2$ ) type. The further inference was therefore drawn that the high ratio type in milk might be derived from grain.

Clark and Lubs (1915) showed that the gas ratio ( $\text{CO}_2 : \text{H}_2$ ) varies inversely with the limiting hydrogen ion concentration attained in standard glucose peptone media containing a standard amount of buffer substance<sup>1</sup>, the low ratio type producing a hydrogen ion concentration equal to

$$1 \times 10^{-5} \bar{N} - 2 \times 10^{-5} \bar{N},$$

and the high ratio type a hydrogen ion concentration equal to

$$7.8 \times 10^{-7} \bar{N} - 0.1 \times 10^{-7} \bar{N},$$

<sup>1</sup> Composition of Clark and Lubs medium: glucose 0.5 %, peptone (Witte) 0.5 %, dipotassium phosphate  $\text{K}_2\text{HPO}_4$  0.5 %.

and showed moreover that these concentrations can be distinguished by means of the indicators methyl-red and *p*-nitro-phenol, and thereby rendered a simple method available for the differentiation of these two main types suitable for routine bacteriological examinations.

Levine (1916) correlated the Voges-Proskauer reaction with the limiting hydrogen ion concentration, having found that organisms producing a low concentration gave the Voges-Proskauer reaction while those producing a high concentration did not, and later, in conjunction with Weldin and Johnson (1917), devised an improvement in the original reaction accelerating the oxidation process with hydrogen peroxide. Myrtle Greenfield (1916) studied 432 cultures of lactose-fermenters from surface water, ground water, natural and artificial ice, and found that 138 (or 32 %) produced a low concentration of hydrogen ions. All these gave the Voges-Proskauer reaction, while none of those cultures producing a high concentration of hydrogen ions gave a positive V.-P. reaction.

Other workers in America have obtained similar results confirming the correlation of the Voges-Proskauer reaction with the limiting hydrogen ion concentration.

For convenience organisms producing high and low concentrations of hydrogen ions will be referred to in this paper as methyl-red positive ("M.R. +") and methyl-red negative ("M.R. -") respectively, and organisms giving and not giving the Voges-Proskauer reaction as "V.-P. +" and "V.-P. -."

As regards the distribution of the "M.R. +" and "M.R. -" types the following additional observations have been recorded:

Levine (1916) examined lactose-fermenters from the faeces of the horse (19), pig (21), cow (20), sheep (22), and man (25) and found none of the "M.R. -" type out of this total of 107.

Rogers, Clark and Lubs (1916) tested 113 cultures from human faeces and found only six (or 5.6 %) of the "M.R. -" type<sup>1</sup>. Rogers (1916) examined 137 cultures from surface water, 66 % of which were found to belong to the "M.R. -" type. The "M.R. +" types were occasionally found in springs in which there was no evident source of contamination, but were especially abundant in polluted waters. Johnson (1916) examined 363 coli-like organisms derived from soil and found 72 % of the "M.R. -" type.

Burton and Rettger (1917) found the predominant gas formers in soil were of the "V.-P. +" type and liquefied gelatine, *i.e.* were of the *B. cloucae* type. Out of 193 non-sporing lactose-fermenters 76 % were "V.-P. +" and liquefied gelatine, 5 % were "V.-P. +" and did not liquefy gelatine, and 19 % were "V.-P. -". He used the M.R. reaction also but regarded the V.-P. reaction as the more reliable with peptones other than Witte's.

<sup>1</sup> In a recent paper Rogers, Clark and Lubs (1918) record the isolation of 46 "M.R. -" strains from a collection of 177 derived from human faeces. The majority of these however were obtained by special methods, and 31 were obtained from a single specimen.



Levine (1918) gives the relative proportion of strains in soil as *cloacae* 49.7 %, *aerogenes* 30.5 %, and "M.R. +" types 18.7 %.

Observations of the Voges-Proskauer reaction have been recorded by a few investigators in this country, and, although it has not been regarded as altogether reliable, it is interesting to compare the results obtained with those briefly summarised above.

MacConkey (1906) recorded the examination of 107 cultures of non-chromogenic lactose-fermenters isolated from milk 31 of which (or 29 %) gave the Voges-Proskauer reaction. The same author (1909) examined 497 cultures from various sources including human and animal faeces, sewage, pond-water, roof washings, soil, and various grains. Organisms giving the Voges-Proskauer reaction were shown to be rare in human and animal faeces (21 in 334 or 6.3 %), more common in sewage and pond-water, roof-washings and soil (32 in 65 or 49 %), and still more common in grain (17 in 30 or 57 %); a fair number in the last two groups were, however, chromogenic.

Orr (1908) examined 850 cultures of glucose-fermenters isolated from milk and found that 333 (or 39 %) gave the Voges-Proskauer reaction. The number of cultures fermenting lactose was not recorded.

Houston (1911) examined 532 lactose-fermenters isolated from water and found that 10.3 % from raw river water, 5.3 % from stored river water, and 3.2 % from stored and filtered river water gave the Voges-Proskauer reaction: no evidence of increase of the "V.-P. +" types after storage.

In India, Clemesha (1912) found that *B. lactis aerogenes* ("V.-P. +") was rare in recently polluted waters but became extremely common within a period of 5-15 days after pollution; that this organism became very common in surface waters after rainy seasons, and that *B. cloacae* ("V.-P. -") was the predominant type after dry seasons. He examined 104 samples of human faeces and 1207 cultures, only 4.6 % of these gave the Voges-Proskauer reaction, while from 86 samples of bovine faeces and 1029 cultures this reaction was only given by 13.4 %.

Recently experiments have been carried out by Winslow and Cohen (1918) on the viability of "M.R. +" and "M.R. -" types in water. The average result of 11 experiments showed a relative increase in the "M.R. -" type after nine weeks storage from 46 % to 71 %. These investigators were unable to find a proportion of the "M.R. -" type in gas-producers isolated from unpolluted or stored waters greater than the proportion found in polluted and unstored waters.

To sum up: the results of various investigators show that the lactose-fermenting bacilli can be divided into two main types by the methyl-red or Voges-Proskauer tests, that the "M.R. -" "V.-P. +" type are rare in the faeces of man and animals, are more common in surface water and sewage, and are the predominant type in grain and soil. These findings are in favour of the view that they are either the natural survivors of the lactose-fermenters present in excretal matter, or are derived from soil, or possibly from grain,

and consequently their presence in water and food products is to be regarded as of less sanitary significance than the presence of excretal *B. coli*.

The object of the present investigation was (1) to add confirmation to the results outlined above (no observations based on the methyl-red test have as yet been recorded in this country), (2) to form some idea of the frequency with which the "M.R. -" type is likely to be encountered in water examinations, etc. using bile-salt media, and (3) to throw new light on the sanitary significance of this type in water and food stuffs by noting the presence or absence of *streptococci* in water samples in which they are found<sup>1</sup>.

The following examinations were made:

- |                                       |                    |
|---------------------------------------|--------------------|
| A. Human and animal faeces.           | C. Milk.           |
| B. Cereals, grain and other articles. | D. Water supplies. |

#### A. THE EXAMINATION OF HUMAN AND ANIMAL FAECES.

The faeces emulsion in sterile water was in most cases plated direct on lactose bile-salt neutral-red agar. In certain cases, however, it was first cultivated in lactose bile-salt broth and then plated out. Subcultures showing gas-production in lactose broth were cultivated in the glucose di-potassium phosphate medium of Clark and Lubs for five days at 37° C., the culture divided into two portions and tested with methyl-red and for the Voges-Proskauer reaction. With the latter, observations were made 24 hours (or within 24 hours if positive) after the addition of the sodium hydroxide.

The following specimens and cultures were examined and gave results as tabulated below:

Table I.

*Cultures from human and animal faeces.*

Source	No. of Cultures	No. of Individuals	Methyl-red		Voges-Proskauer	
			+	-	+	-
Human faeces	33	11	33	0	0	33
Horse ..	17	8	16	1	0	17
Cow ..	13	7	13	0	0	13
Sheep ..	20	7	20	0	0	20
Rabbit ..	18	6	15	3	3	15
Mouse ..	25	7	22	3	4	21
Cat ..	3	1	3	0	0	3
Guinea-pig faeces	3	1	3	0	0	3
Total	132	48	125	7	7	125
Percentage	—	—	94.7	5.3	5.3	94.7

#### Notes on Table I.

1. The three "M.R. -" strains from rabbit faeces were from the same individual: a tame rabbit fed on oats.

2. The three "M.R. -" "V.-P. +" strains from mouse faeces and the one "M.R. +" "V.-P. -" strain were from three individuals, all wild mice.

3. "M.R. -" strains (not included in the above series) were isolated from two specimens of cow faeces as the ultimate survivors in water after several weeks' storage in the course of some experiments on the relative viability of *B. coli* and *Streptococci* [Savage and Wood (1918)].

<sup>1</sup> It has been shown by Savage and Read (1916) that *streptococci* are to be found in the majority of waters subject to contamination, and by Savage and Wood (1918) that these organisms die out rather more rapidly than *B. coli* and are useful indicators of recent contamination.

The above represent only a limited number of specimens and cultures, but a sufficient number to confirm the findings of other workers, viz. that the "M.R. —" "V.-P. +" type is very rare in human and animal faeces. It has been noted that organisms of the "M.R. —" type frequently gave a less definite reaction on neutral-red lactose bile-salt agar than those of the "M.R. +" type, the paler colonies were accordingly selected when present in order to favour the isolation of the "M.R. —" type as much as possible.

The correlation between the methyl-red and Voges-Proskauer tests was not quite perfect, though very nearly. Some cultures give neutral tints with methyl-red and a positive Voges-Proskauer reaction when peptone other than Witte's is used for the standard medium. Both tests should therefore be applied. The supply of Witte's peptone was exhausted when the above tests were made and Baird and Tatlock's "Bactopeptone" was used and found to be a very good substitute.

Table II.

*Cultural characters of "M.R. —" types isolated from faeces.*

Source	No.	Gas in Lactose	Gas in Saccharose	Litmus milk		Indol	Character of growth on gelatine	Liquefaction of gelatine in 14 days	Voges-Proskauer reaction	Production of capsule
				acid	clot					
Rabbit	4	$\frac{3}{4}$ in.	$1\frac{1}{2}$ in.	+	+	+	...	+	+	+
"	5	$\frac{1}{2}$ "	$1\frac{1}{2}$ "	+	+	+	...	+	+	+
"	6	$\frac{5}{8}$ "	$1\frac{1}{2}$ "	+	+	+	...	+	+	+
Mouse	9	$\frac{3}{8}$ "	$1\frac{1}{2}$ "	+	+	+	...	+	+	+
"	10	$\frac{3}{4}$ "	2 "	+	+	—	...	—	+	+
"	11	$\frac{1}{4}$ "	2 "	+	+	—	...	+	+	+
Cow	1	$\frac{1}{2}$ "	$1\frac{1}{2}$ "	+	+	—	opaque	—	...	+
"	2	$\frac{1}{2}$ "	$\frac{3}{4}$ "	+	+	—	"	—	...	+
"	3	slight	$1\frac{1}{2}$ "	+	+	—	"	—	...	+
"	7	$\frac{1}{2}$ in.	$1\frac{1}{2}$ "	+	+	—	"	—	...	+

*Notes on Table II.*

1. All cultures fermented saccharose and most of them with abundant gas production.
2. All cultures showed capsule formation in milk.
3. Quite a large proportion produced indol.

## B. THE EXAMINATION OF CEREALS, GRAIN AND OTHER ARTICLES.

Sixty specimens were examined, made up as follows:

*Whole grain.* Oats 12, wheat 14, barley 11.

*Flours, etc.* Crushed oats 6, barley flour 2, wheat flour 2, maize flour 4, rice flour 1.

*Other articles.* Straw 2, dried milk 3, egg powder 1, hay 3.

A small quantity was cultivated in lactose bile-salt broth and when acid and gas were produced, plated out on lactose bile-salt agar with neutral-red. In some cases the grains were first allowed to germinate in a sterile moist chamber at 21° C.

Lactose-fermenters were isolated from 20 specimens, from 16 of these only "M.R. -" strains were obtained, while in the remaining four only "M.R. +" strains were found.

The specimens from which the "M.R. -" strains were isolated together with their cultural characters are given in Table III.

Table III.

"M.R. -" strains isolated from cereals, grain, and other articles.

No of specimen	Source	No. of culture	Gas in Durham's tube in inches		Litmus milk		Indol	Character of growth on gelatine	Liquefaction of gelatine in 14 days	Voges-Proskauer reaction	Production of capsule in milk
			Lactose	Saccharose	acid	clot					
1	Dried milk	1	$\frac{3}{4}$	$\frac{3}{4}$	+	+	-	opaque*	-†	...	+
		2	$\frac{1}{4}$	$1\frac{1}{4}$	+	+	-	translucent*	-†	...	+
		3	$\frac{1}{8}$	$1\frac{1}{4}$	+	+	-	"	*	-†	+
2	"	4	bubble	$\frac{5}{8}$	+	+	-	"	*	-†	+
		5	$\frac{3}{8}$	$1\frac{1}{4}$	+	+	+	"	*	-†	+
3	Egg powder	6	$\frac{1}{2}$	2	+	+	-	"	-†	+	+
4	Crushed oats	7	$\frac{1}{2}$	$\frac{1}{2}$	+	+	-	"	-†	+	+
		8	bubble	$\frac{3}{8}$	+	+	-	"	*	-†	-
		9	$\frac{1}{2}$	$\frac{1}{2}$	+	+	-	"	-†	+	-
5	Barley flour	10	bubble	$\frac{3}{8}$	+	+	-	"	-	...	...
6	"	11	$\frac{5}{8}$	$1\frac{1}{4}$	+	+	-	"	-	+	-
7	Maize flour	12	1	$\frac{1}{8}$	+	+	+	"	-	+	+
8	"	13	$\frac{1}{8}$	$1\frac{1}{2}$	+	+	-	"	*	+	-
9	Wheat flour	14	$\frac{1}{4}$	$1\frac{1}{2}$	+	+	-	"	+	+	-
10	Straw	15	$\frac{1}{4}$	$\frac{1}{2}$	+	+	-	"	+	...	...
11	Oats	16	$\frac{3}{4}$	$\frac{3}{4}$	+	+	-	"	-	+	-
12	"	17	$\frac{3}{4}$	1	+	+	-	"	-	+	+
13	"	18	$\frac{1}{4}$	...	+	+	-	"	-	...	-
14	Wheat	19	$\frac{3}{8}$	1	+	+	-	"	-	+	+
15	Barley	20	$\frac{1}{2}$	1	+	+	slow	"	+	+	+
16	"	21	$\frac{1}{2}$	2							

\* These cultures produced a slight yellow pigment.

† These cultures liquefied gelatine very slowly.

It will be noticed from Table III that the "M.R. -" strains isolated from cereals and grain frequently showed very weak lactose fermentation, less than  $\frac{1}{4}$  inch of gas being produced and this sometimes only after several days. After cultivation on gelatine or in milk at 21° C. this faculty can be revived as the following instances showed:

	Gas produced in lactose broth	
	before cultivation at 21° C.	after cultivation at 21° C.
Barley flour, No. 10	$\frac{1}{8}$ inch	$\frac{1}{2}$ inch
Maize flour, No. 13	$\frac{1}{8}$ "	$\frac{7}{8}$ "
Wheat flour, No. 19	$\frac{1}{4}$ "	over 1 "
Crushed oats, No. 9	$\frac{1}{4}$ "	$\frac{3}{4}$ "

Grain which had been allowed to sprout in a moist chamber at 21° C. usually showed good fermentation of lactose. The "M.R. -" strains also became very numerous under these conditions, and it is quite conceivable

that surface water in the neighbourhood of grain fields might be considerably affected by germinating grain.

A number of cultures liquefied gelatine, but except in three instances too slowly for this test to be of any diagnostic value. A fair number of cultures were chromogenic, but the majority showed no obvious pigment on gelatine slopes. Saccharose was nearly always fermented and frequently with abundant gas production.

Apparently some organisms of the "M.R. —" type possess remarkable viability in grain and flours. The sample of maize flour No. 7 was re-examined after keeping in a sterile bottle for three months and organisms of the "M.R. —" type were again found.

Excluding all cultures giving less than  $\frac{1}{4}$  inch of gas in lactose (but including cultures Nos. 10, 13, 19 and 9), all cultures liquefying gelatine in 14 days or producing pigment, 12 out of the 21 cultures could not certainly be distinguished from *B. coli* of faecal origin except by the methyl-red and Voges-Proskauer tests, though the reaction in litmus lactose broth and on neutral-red lactose agar frequently suggested that they belonged to the "M.R. —" type.

#### C. EXAMINATIONS OF MILK.

Thirty-two samples of milk were examined and 94 lactose-fermenters isolated. Seventeen of these were found to belong to the "M.R. —" type and 77 to the "M.R. +" type.

In the districts from which these samples were obtained the cows are kept and milked in open fields except in the coldest months of the year when they are brought into sheds. It is interesting to compare the "field" samples with the "shed" samples. Only 8.3% of the former contained "M.R. —" strains while in 28% of the shed samples this type was found. These results support the suggestion of Rogers, Clark and Evans that the "M.R. —" types found in milk have their origin in grain and straw, but the possibility that the colder weather favoured the predominance of this type must be borne in mind<sup>1</sup>.

The cultural characters of the "M.R. —" strains isolated are given in Table IV.

Table IV.

*Cultural characters of "M.R. —" strains isolated from milk.*

Indol production	Litmus milk acid and clot	Gelatine			Capsule production in milk	Voges- Proskauer reaction
		liquefaction	opaque creamy growth	translucent growth		
10 %	90 %	10 %	40 %	60 %	90 %	100 %

No cultures showed obvious pigment on gelatine slopes.

<sup>1</sup> In a recent paper Rogers, Clark and Lubs (1918) have shown that the "M.R. —" types tend to outgrow the "M.R. +" types when milk is allowed to curdle at 20° C.

## D. THE EXAMINATION OF WATER SUPPLIES.

These comprise a large number of samples from various sources, 200 of which contained lactose-fermenters. The results are given in Tables V and VI, the former were from sources of good repute and the latter from miscellaneous sources.

For the enumeration of *Streptococci* the method of Savage was employed: cultivation in glucose neutral-red broth and examination of hanging drop preparations after 48 hours, in doubtful cases examining stained films with the  $\frac{1}{12}$  inch objective.

Table V.

*Methyl-red negative organisms encountered in the routine examination of 200 water samples containing lactose-fermenting organisms.*

No. of sample	Description of source	Sources of good repute			Identification number of "M. R. -" cultures
		Methyl red negative type found in	Methyl-red positive type + = present - absent	<i>Streptococci</i> + = present - absent	
A. 1620	Well (public supply)	10 c. c.	- 40 c. c.	- 40 c. c.	W. 5
A. 1711	do.	30 "	- 40 "	- 40 "	W. 17
1	Deep well in limestone (public supply)	100 "	- 100 "	- 100 "	W. 19
2	do. do. do.	100 "	- 100 "	- 100 "	W. 20
3	do. do. do.	100 "	+ 100 "	- 100 "	W. 22
x. 1748	Well in field, no source of contamination	30 "	- 40 "	- 40 "	W. 23
4	Deep well in limestone (public supply)	100 "	- 100 "	- 100 "	W. 25
5	do. do. do.	100 "	- 100 "	- 100 "	W. 26
B. 1758	Deep well in limestone	10 "	- 40 "	- 40 "	W. 27
6	Deep well in limestone (public supply)	100 "	- 100 "	- 100 "	W. 29
C. 1809	Deep well sunk through Keuper Marl into Sandstone	10 "	- 40 "	- 40 "	W. 41
7	Deep well in limestone (public supply)	1 "	- 100 "	- 100 "	W. 45, 46, 47
8	do. do. do.	1 "	- 100 "	- 100 "	W. 48, 49, 50
9	do. do. do.	100 "	- 100 "	- 100 "	W. 51
10	do. do. do.	100 "	- 100 "	- 100 "	W. 52
11	do. do. do.	100 "	- 100 "	- 100 "	W. 53
12	do. do. do.	100 "	- 100 "	- 100 "	W. 54
13	do. do. do.	100 "	- 100 "	- 100 "	W. 55
14	do. do. do.	100 "	- 100 "	- 100 "	W. 56
15	do. do. do.	10 "	- 100 "	- 100 "	W. 57, 58
16	do. do. do.	10 "	- 100 "	- 100 "	W. 60, 61
17	do. do. do.	10 "	- 100 "	- 100 "	W. 62, 63
18	do. do. do.	10 "	- 100 "	- 100 "	W. 64, 65
19	do. do. do.	100 "	- 100 "	- 100 "	W. 66
20	do. do. do.	100 "	- 100 "	- 100 "	W. 67
21	do. do. do.	10 "	- 100 "	- 100 "	W. 68
22	do. do. do.	100 "	- 100 "	- 100 "	W. 69
1823	Spring	30 "	- 40 "	- 40 "	W. 70

Table VI.

*Methyl-red negative organisms encountered in the routine examinations of 200 samples of water containing lactose-fermenting organisms.*

Miscellaneous sources.					
No. of sample	Description of source	Methyl-red negative type found in	Methyl-red positive type +=present -=absent	<i>Streptococci</i> +=present -=absent	Identification number of "M.R. -" cultures
1485	Shallow well	30 c.c.	-40 c.c.	-40 c.c.	W. 1
1532	do.	10 "	-40 "	-40 "	W. 2
1610	do.	$\frac{1}{16}$ "	...	- $\frac{1}{16}$ "	W. 3
1618	Deep well	1 "	...	+10 "	W. 4
1622	Shallow well	1 "	...	+1 "	W. 6
1642	do.	$\frac{1}{16}$ "	...	+1 "	W. 7
1643	do.	10 "	...	-40 "	W. 8
1652	do.	10 "	...	+30 "	W. 9
1658	do.	10 "	...	+30 "	W. 10
1694	Deep well	10 "	-40 "	-40 "	W. 12
1695	Shallow well	$\frac{1}{16}$ "	+1 "	+1 "	W. 13
1706	do.	$\frac{1}{16}$ "	+ $\frac{1}{16}$ "	+1 "	W. 14
1709	do.	10 "	+10 "	+1 "	W. 16
1712	do.	10 "	+30 "	+10 "	W. 18
1734	do.	10 "	+30 "	-40 "	W. 21
1691	Spring	30 "	-40 "	-40 "	W. 11
1751	Shallow well	10 "	...	+30 "	W. 24
1760	do.	10 "	...	+10 "	W. 28
1792	do.	1 "	+10 "	+30 "	W. 30
1793	do.	30 "	-40 "	-40 "	W. 31
1799	Spring	30 "	-40 "	-40 "	W. 32
1800	do.	30 "	-40 "	-40 "	W. 33
1802	do.	30 "	-40 "	-40 "	W. 34
1803	do.	30 "	-40 "	-40 "	W. 35
1804	do.	1 "	-40 "	-40 "	W. 36, 37, 38
1807	do.	30 "	-40 "	-40 "	W. 39
1808	Well	30 "	-40 "	-40 "	W. 40
1810	Spring	30 "	-40 "	-40 "	W. 42
1813	Well	1 "	...	-40 "	W. 43
1814	do.	1 "	+30 "	+30 "	W. 44
1821	Shallow well	10 "	...	+10 "	W. 59
1828	do.	1 "	+10 "	+30 "	W. 73
1836	do.	1 "	+10 "	-40 "	W. 74
1855	do.	$\frac{1}{16}$ "	...	+10 "	W. 75
1857	do.	1 "	+10 "	+30 "	W. 76
1858	do.	$\frac{1}{16}$ "	...	+ $\frac{1}{16}$ "	W. 77
1870	Spring	10 "	...	+10 "	W. 78

When *Streptococci* and "M.R. -" strains (the latter in 10 c.c. or less) were both present the evidence of contamination was generally considered sufficiently proven, and the presence of "M.R. +" strains in larger quantities was not as a rule sought. In samples Nos. 1695, 1706, 1709, 1712, 1734, 1792, 1814, 1828, 1836 and 1857 the evidence of contamination, as judged by the presence of both "M.R. -" strains and *Streptococci*, was confirmed by the isolation of "M.R. +" strains from a larger quantity of the sample.

It will be noticed that 29 of the samples (Table V) were from sources of good repute. In only one of these were organisms of the "M.R. + " type also found, and *Streptococci* were found in none.

Thirteen samples from miscellaneous sources contained "M.R. - " strains but no "M.R. + " strains or *Streptococci*.

Out of the total of 66 samples, therefore, in which "M.R. - " strains were found, as many as 41 contained no "M.R. + " strains or *Streptococci*. Judgment of the water was therefore subject to modification in 62 % of these samples, the results suggesting that no recent excretal contamination had occurred.

Of special interest was the occurrence of organisms of the "M.R. - " strain in the deep wells (numbered 1 to 22). This supply which comprises several wells, sunk through limestone some 250-400 feet into underlying sand, has been kept under observation for several years, and the bacteriological results have been very good. Occasionally, in some 15 % of all samples examined, lactose-fermenters have been found, but seldom in less than 100 c.c., and these have nearly always failed to produce indol. In the spring of 1918 lactose-fermenters (all "Lactose + Indol - ") were found in a larger proportion of samples, and the application of the methyl-red and Voges-Proskauer tests showed that these with one exception were of the "M.R. - " "V.-P. + " strain. On no occasion were *Streptococci* found. The simultaneous appearance in all the wells, which are several miles apart, is difficult to account for. The time of the year rather suggests some connection with the sowing of grain. Rogers, Clark and Lubs (1918) have recently shown that the majority of "M.R. - " strains derived from grain do not ferment adonitol. Unfortunately a supply of this alcohol was not available and tests could not be made.

#### CULTURAL CHARACTERS OF "M.R. - " STRAINS ISOLATED FROM WATER.

*Lactose litmus peptone.* Eleven cultures (or 18 %) produced  $\frac{1}{4}$  inch or less of gas in the Durham's tube. The litmus indicator frequently showed less acidity than that given by "M.R. + " strains.

*Saccharose.* Only 27 cultures were tested. All fermented saccharose and many with remarkable gas production, the Durham's tube being completely filled in some instances.

*Gelatine.* Liquefaction was observed with six (or 9 %) but only with three was it sufficiently rapid to be of diagnostic value.

None showed obvious pigment.

25 % showed the opaque creamy growth typical of *B. lactis aerogenes*, the rest being more or less translucent.

*Capsule production in milk.* 84 % produced capsules.

*Litmus milk.* 95 % showed acid and clot within a week.

*Indol.* 15 % produced indol in five days.

*Voges-Proskauer reaction.* 92 % gave this reaction.

*Lactose bile-salt neutral-red agar.* Colonies were frequently somewhat paler



than "M.R. +" organisms. The large majority developed mucoid colonies with tendency to become confluent.

English standards as to what is to be considered an excretal type of *B. coli* as distinguished from a coliform organism vary to some extent. Such organisms are required in this laboratory to have the following characters, and it may be taken that this definition would be accepted by most bacteriologists:

*Lactose.* Acid and gas production (a bubble or less than  $\frac{1}{4}$  inch in the Durham's tube excluded)<sup>1</sup>.

*Litmus milk.* Acid and clot production within seven days.

*Gelatine.* No liquefaction within two weeks, and no pigment formation.

*Indol.* Not necessarily produced, but a fairly strong point against its being of recent excretal origin if not produced.

The value of the methyl-red and Voges-Proskauer tests really depends upon the extent to which they are capable of further differentiating organisms *with the above characters* into two groups, one of which is truly excretal and the other non-excretal in origin or very resistant. From this point of view only organisms possessing the above characters need be considered. The following table includes only such organisms:

Table VII.

Material	Number of strains tested	Number of strains			Percentages	
		"M R. +"	"M.R. -" Indol +	Indol -	"M R. +"	"M.R. -"
Human faeces	33	33	0	0	100	0
Animal faeces	99	91	4	4	91.9	8.1
Cereals and grain	15	4	1	10	26.5	73.5
Water	231	154	12	65	66.7	33.3
Milk	93	77	2	14	82.5	17.5

The Committee appointed by the Council of the Royal Institute of Public Health in 1914 recommended that in the bacterioscopic examination of waters reports should be based upon the enumeration of "Lactose + Indol + " organisms. If as strict a definition as this be adopted it is evident that only 19 strains from the above series would come under consideration. In judging the purity of a supply from an individual sample, or even from a limited number of samples, however, it is not possible to take so strict a line. In the author's experience a water supply to which an outbreak of typhoid fever was definitely traced yielded only Lactose + Indol - organisms in the first three examinations together with *Streptococci*, subsequent examinations yielding Lactose + Indol + organisms. In the above series of water examinations 13.5 % of the lactose-fermenters did not produce indol and did *not* belong to the "M.R. -" type. Moreover, some of the Lactose + Indol + organisms belong to the "M.R. -" type, Levine (1918) recorded as many as 25 % producing indol.

<sup>1</sup> Some bacteriologists use gelatine shake cultures for observation of lactose fermentation. This is extremely sensitive and cultures producing only a bubble of gas in the Durham's tube would be recorded as positive.

## SUMMARY AND CONCLUSIONS.

1. Investigations by American bacteriologists have shown that the lactose-fermenting (gas-producing) bacilli can be divided into two main types distinguishable by the methyl-red and Voges-Proskauer reactions.

2. The methyl-red - Voges-Proskauer + type are shown to be rare in human and animal faeces, more common in surface water, milk and sewage, and the predominant type in soil and grain, and to be more resistant than the methyl-red + Voges-Proskauer - type.

3. Investigations by the author of this paper confirm their findings as regards human and animal faeces, water, milk and grain. An investigation of the types present in soil is being undertaken and it is hoped to publish an account of this later. Already the "M.R. -" type has been found to predominate in four out of six samples of soil.

4. In the present investigation organisms of the "M.R. -" type were found in 66 samples of water out of a total of 200 containing lactose-fermenters, and in 41 samples containing this type no evidence of recent contamination could be adduced by the search for organisms of the "M.R. +" type or *Streptococci*. Judgment of the water was therefore liable to modification in 20 % of these samples by the recognition of this type.

5. Twenty-nine out of the 66 samples containing organisms of the "M.R. -" type were from sources of good repute, most of them from public supplies.

6. The presence of lactose-fermenters of the "M.R. -" "V.-P. +" type must be regarded with considerably less disfavour than the presence of "M.R. +" "V.-P. -" organisms, and the application of tests for the recognition of these types is important. It is suggested that these tests should be included in all routine examinations of water and food products.

In conclusion I have pleasure in acknowledging my indebtedness to Dr W. G. Savage for calling my attention to the researches of American bacteriologists and for many valuable suggestions he has made in connection with this work.

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# THE CULTIVATION OF *SPIROCHAETA ICTEROHAEMORRHAGIAE* AND THE PRODUCTION OF A THERAPEUTIC ANTI-SPIROCHAETAL SERUM.

By A. STANLEY GRIFFITH, M.D.

(*Report to the Medical Research Committee.*)

(*From the Field Laboratories, University of Cambridge.*)

## INTRODUCTION.

THE Japanese investigators (Inada and Ido) who discovered the *Spirochaeta icterohaemorrhagiae*, the cause of infectious jaundice, showed that the serum of patients who had recovered from the disease contained immune substances which were capable of destroying the spirochaetes in the blood and tissues of an experimentally infected guinea-pig. They found that if the serum were injected into the guinea-pig prior to the appearance of icterus the disease was inhibited in all cases. The same result was obtained with the serum of immunised goats.

On the basis of this work Inada and Ido advanced the hypothesis that serum therapy should be effective in the human disease.

In the treatment of human cases they tried in the first instance the serum of recovered human cases and later the serum of actively immunised horses.

As the result of these first trials they came to the conclusion that the administration of the immune serum had a beneficial effect in cases of the disease.

Cases of jaundice with the clinical course of infectious jaundice were first observed among the troops on the Western front in the summer and autumn of 1915 but it was not until 1916, following the publication of the Japanese work, that *Spirochaeta icterohaemorrhagiae* was demonstrated in the blood and urine of the affected persons (Stokes and Ryle, 1916). The spirochaete was also found by Adrian Stokes (1917) in rats captured in the trenches where the cases of jaundice had originated.

In view of the favourable reports by the Japanese of the serum treatment of infectious jaundice and of the possibility of further cases arising among the British troops, seeing that the parasite was being harboured by the trench rat, it was decided by the Medical Research Committee to supply a curative serum for the treatment of cases of the disease.

The work of preparing the serum was entrusted to me and was begun in February 1917.

I am indebted for my original material to Dr J. MacIntosh who sent me a portion of the liver of a guinea-pig which had died of experimental spirochaetosis. The strain of spirochaete used was that known as the Belgian strain and had been originally obtained by Captain Adrian Stokes from a case of infectious jaundice.

This strain had not at that time been brought into artificial cultivation and it was decided after discussing methods with Dr H. H. Dale to begin the immunisation of a horse with tissue spirochaetes and to continue with culture if cultivation experiments were successful.

My work on the subject therefore can be described under two heads dealing respectively with (a) the immunisation of horses with the spirochaete and (b) the artificial cultivation of the spirochaete.

It will be convenient to record first the cultivation experiments.

#### BIOLOGICAL CHARACTERISTICS.

##### ARTIFICIAL CULTIVATION OF *SPIROCHAETA ICTERHAEMORRHAGIAE*.

The Japanese observers, Inada and Ido, succeeded in cultivating *Spirochaeta icterohaemorrhagiae* by the method of Noguchi for the culture of the spirochaete of recurrent fever. I tried this method with various kinds of animal sera and human ascites fluid to which had been added, as recommended by Inada and Ido, guinea-pig kidney instead of rabbit kidney. The media were inoculated with pieces of the liver of an infected guinea-pig and were incubated at 37° C. and 25° C. both under aerobic and anaerobic conditions.

My experiments with these fluid media, like those of Adrian Stokes with similar media, were unsuccessful.

I then tried diluted bovine serum (serum 1 part and physiological salt solution 2 parts) which had been heated to 70° C. until it had become slightly viscous and I inoculated the tubes with the heart blood as well as with fragments of the liver of an infected guinea-pig. In this medium incubated at 25° C. a growth of the spirochaete was obtained at the first attempt and I was able subsequently to transmit the organism through several generations. Shortly after obtaining the first culture I had the opportunity, through the courtesy of the Secretary of the Medical Research Committee, of reading an advance proof of Noguchi's paper (1917) in which he reported the cultivation of the Belgian as well as the French and American strains of the spirochaete.

Noguchi grew the spirochaete in a fluid medium in which there were loose strands of fibrin produced by adding a small quantity of citrate plasma to the diluted or undiluted serum of a suitable animal. He recommended for routine use two media which had given equally satisfactory results:

(a) Rabbit serum (1 part) + Ringer's solution or 0.9 per cent. sodium chloride solution (3 parts) + citrate rabbit plasma (0.5 part) covered with a thin layer of sterile paraffin oil; (b) the same, except for the use of 0.5 to 1.0 part of neutral or slightly alkaline

agar (2 per cent.) which should be added while in a liquid state and quite hot (60–65° C.) in order to get a uniform mixture of the agar.

I was able to cultivate the spirochaete in these media but I obtained more luxuriant initial cultures in diluted serum which had been previously heated until it had become viscous or semi-gelatinous. This kind of medium was therefore adopted for the routine primary cultivation of the spirochaete.

The sera used were those of the horse, rabbit and adult cattle. Of these bovine serum has given the most consistently good results and has been most frequently employed. Bovine serum was also found by Martin, Pettit and Vaudremer (1917) to be very suitable for the cultivation of the spirochaete. Horse serum in a semi-gelatinous state proved less favourable for the growth of the spirochaete than semi-gelatinous bovine or rabbit serum, but horse serum unheated and mixed with 0.5 per cent. agar was found to be not inferior in nutritive value to bovine and rabbit serum.

Very satisfactory results have also been obtained with a medium composed of 1 part of the citrated blood of the horse, cow or rabbit mixed with 1 or 2 parts of physiological saline and heated until semi-gelatinous. In the horse blood medium the spirochaete sometimes grew extremely luxuriantly and one strain was transmitted through many subcultures in this medium. Ito and Matsuzaki (1916) recommended the use of a blood gelatin or a blood agar medium.

Noguchi (1918), in a recent paper, has recorded the results of his investigations of the value of different animal sera for the cultivation of the spirochaete. He found that the cultural value of different animal sera varies considerably. It is entirely absent from the sera of the rat and the pig. The sera of the rabbit, horse and goat are better suited for the growth of the organism than those of the guinea-pig, sheep, donkey or calf. Human serum is suitable but not ascites fluid. Fresh and heated emulsions of organs and the white and yolk of hens' eggs have no cultural value. (I also tried an egg medium, and, like Noguchi, failed to get a growth of the spirochaete.)

Noguchi found further that the nutritive value of serum is considerably reduced by heating to 60° C. for 30 minutes. My few comparative observations bearing on this latter point support Noguchi's finding. It has been observed that the addition of a small quantity of the citrated blood of a normal guinea-pig increases the nutritive value of a heated serum medium, thus indicating that the fresh blood restored to the medium something which was lost in the process of heating. This enrichment was specially noted with horse and rabbit serum. Bovine serum on the other hand, especially when of a golden yellow colour in the fluid state, showed little diminution in nutritive value after heating and produced luxuriant subcultures without any addition of fresh blood. With guinea-pig blood added semi-gelatinous bovine serum is in my experience an excellent medium for the cultivation of the spirochaete and by its use I have kept a strain of the organism in artificial cultivation for more than 18 months.

For raising initial cultures from an infected guinea-pig the citrated heart blood is the most convenient and suitable material. Liver and even kidney may be used but the cultures are more liable to become contaminated with other organisms than when blood is the material employed. Subcultures are made by transferring with a sterile pipette a few drops from the top of the medium to the surface of the fresh medium. All media are covered with a thin layer of sterile paraffin oil.

The spirochaete is an obligatory aerobe and grows best as Noguchi has also observed in the upper centimetre of the medium. When the medium is gelatinous spirochaetes are found only in small numbers in the middle and often not at all in the deep parts of the tube. In fluid media on the other hand there is wider distribution of the organism throughout the medium, possibly because oxygen, as Noguchi suggests, is able to penetrate more deeply into a fluid than into a semi-solid medium.

In semi-gelatinous media the spirochaetes are at first unevenly distributed in the upper layer. While some areas are colonised by enormous numbers of the organism others contain only moderate numbers and others again very few or none.

The spirochaete has been cultivated by me at two temperatures only, namely at 37° C. and 25° C. It is stated that it also grows at lower temperatures, down to 10° C.

Multiplication is as a rule more rapid at 37° C. than at 25° C. but sometimes, in primary cultures, growth has been as rapid at 25° C. as at 37° C.

After two days' incubation at 37° C. (primary culture, semi-gelatinous serum medium) spirochaetes may be found in swarms in the upper centimetre of the medium. At the end of a week they are less numerous and degenerate forms are seen. In a fortnight they have diminished in numbers very considerably and there are many granules, the remains of degenerated spirochaetes. In the fourth week they have often disappeared completely from the medium which is no longer capable of infecting the guinea-pig. In order therefore to maintain a culture of the spirochaete at 37° C., subcultures should be made at short intervals. I did not however in my early experiments succeed in transmitting the spirochaete through more than five generations at this temperature and this result supported the statement of Inada and Ido (1916) that 37° C. is unfavourable for the cultivation of the organism. Recently I have had better success at this temperature, using a strain of the spirochaete which had been in cultivation for more than a year at 25° C.; this strain has been for several generations and is now growing luxuriantly at 37° C.

At 25° C. growth is in general not so rapid as at 37° C. but the organism retains its vitality for a longer period and can be subcultured through many generations. In subcultures at 25° C. spirochaetes do not as a rule become numerous until the second or the third week. When the medium is semi-gelatinous the spirochaetes begin after a time (two to three months) to degenerate and gradually to disappear while in more fluid media, though they

may diminish in number, they persist and remain viable for indefinite periods.

The Japanese observers, Inada and Ido (1916), stated that the life of a culture is variable.

The first generation lived mostly from three to six weeks, the longest period observed being 55 days and the shortest 17 days. The life of the 2nd and 3rd generation is somewhat shorter than that of the first generation. The best time for transferring the culture from one tube to another is when multiplication is going on rapidly as indicated by examination of the fluid every two or three days.

My observations, while confirming the statement that the length of life of individual cultures varies, show that in a suitable medium the organism may live for very long periods not only in primary but also in secondary cultures.

Transference of the cultures have usually been made at intervals of from two to six weeks but subcultures up to 16 weeks old have grown luxuriantly in fresh tubes of medium. In fact there seems to be no limit to the age of a culture for successful subcultivation; if spirochaetes are present in the medium they will grow in a suitable new medium no matter what their age.

In order to ascertain how long a culture of the spirochaete would remain viable the first primary culture has been preserved (at 25° C.) and has been tested at intervals, the last test being made when the culture was 15 months old. On every occasion the spirochaetes were found to be capable of growing vigorously and luxuriantly in subculture.

No final statement therefore can be made at present as to the maximum duration of life of the spirochaete outside the body in a favourable medium. The primary culture is being preserved and will be tested again after a more prolonged interval.

#### PATHOGENICITY OF CULTURES.

Young recently isolated cultures of the spirochaete produce the same morbid effects in the guinea-pig as tissue spirochaetes. The pathogenicity of the cultivated spirochaete however appears quickly to be lost. A culture grown at 37° C., which, when a fortnight old produced typical spirochaetosis in a guinea-pig, lost its virulence within the next fortnight (two experiments). In another experiment with spirochaetes grown at 25° C., a 14 days' old primary culture produced fatal haemorrhagic jaundice while the same culture when 3½ and 4 months old was completely non-pathogenic. A 14 days' old subculture from the four months' old primary culture was also devoid of virulence for the guinea-pig.

The guinea-pigs which failed to develop spirochaetosis after the injection of living cultures were after intervals of a month inoculated with emulsion of liver from a fatally infected guinea-pig. The guinea-pigs were entirely unaffected though control guinea-pigs receiving the same amounts of emulsion died in three to four days of acute spirochaetosis.



The above observations show that the virulence of the spirochaete for the guinea-pig is soon lost in culture and that the attenuated cultures of the organism are capable of inducing an active immunity in guinea-pigs.

Attempts to raise the virulence of the attenuated cultures by passage through the bodies of very young guinea-pigs, rats, mice and toads have not so far been successful.

The rats and mice were killed two to four weeks after intraperitoneal injection of the cultures and their kidneys were emulsified and injected into guinea-pigs. No spirochaetes were found in the emulsions, or in smear preparations of the spleen, liver and blood, and the guinea-pigs remained unaffected.

Toads (three experiments) were used with a view to ascertaining whether the spirochaete is capable of multiplying in the bodies of cold blooded animals. No spirochaetes were found in smear preparations of their organs, heart blood or subcutaneous lymph 14 and 28 days after inoculation.

#### THE MORPHOLOGY OF THE SPIROCHAETE AND THE PATHOGENIC EFFECTS IN THE GUINEA-PIG.

My observations on the morphology of *Spirochaeta icterohaemorrhagiae* and on the gross pathological appearances of experimental spirochaetosis in the guinea-pig agree with those of other workers (Noguchi, 1918; Stokes and others, 1917; Inada and others, 1916) and I have nothing new to add to what has already been published in these connections.

#### THE IMMUNISATION OF HORSES.

##### *Horse 1.*

On 6 February, 1917, the immunisation of a horse was begun. As stated in the introduction it was decided to use as antigen in the first place emulsions of the livers of guinea-pigs which had died of experimental spirochaetosis and to continue with cultures if cultivation experiments were successful.

During the first four weeks the horse received seven injections of liver emulsions which had been sterilised by heat (at first 55° C. and subsequently 50° C.) or by the action of 0.5 per cent. phenol. The injections were made intramuscularly, the first dose being 5 c.c. of a thick emulsion, the seventh 20 c.c. containing the emulsifiable tissue of the livers of two young guinea-pigs.

After the latter injection the serum was found by Dr J. MacIntosh to have definite agglutinative and lytic action on living spirochaetes and it was considered therefore that the administration of the living organism might safely be begun.

The first dose of the living virus was 5 c.c. of liver emulsion rich in spirochaetes. The doses were thereafter gradually increased up to 25 c.c. containing the emulsifiable tissue of the livers of three guinea-pigs. As cultures of the spirochaete were now available the next dose (or tenth dose of living

virus) was partly liver emulsion and partly culture. Unfortunately only one guinea-pig was available and, as the cultures were only moderately luxuriant, this dose of antigen was therefore less rich in spirochaetes than the previous ones.

With a view to the speedy attainment of a high titred serum it was decided at this stage to continue the immunisation by the intravenous inoculation of cultures.

20 c.c. of a fluid culture containing numerous spirochaetes were accordingly injected into the jugular vein on June 15. Shortly after the injection the horse began to show signs of distress; then clonic contractions of the muscles set in and the horse fell down unconscious; respiration continued for a few moments when death ensued.

#### TESTS OF THE POTENCY OF THE SERUM.

The titre of the serum was determined at various stages of the immunisation by ascertaining the quantity of serum which would suffice to protect a guinea-pig weighing from 200 to 300 grams from a fatal dose of spirochaetes.

The test dose of virus was 1.0 c.c. of an emulsion of guinea-pig liver rich in spirochaetes. The doses were not standardised but the emulsions used were approximately of equal density. Though there was no doubt considerable variation in the numbers of spirochaetes injected in the different sets of experiments this variation did not appear to affect materially the comparative value of the tests.

The serum was injected a few minutes after the virus. Both virus and serum were injected intraperitoneally.

Three samples of the serum were tested. The first taken after about two months' immunisation gave a titre of 0.25 c.c., *i.e.* this amount of serum completely protected a guinea-pig from the test dose of spirochaetes. The second sample (May 11) had a titre of 0.1 c.c., while the third (June 14) was rather less potent than the second, 0.1 c.c. failing to protect the guinea-pig from death though it considerably prolonged the duration of life. The fall in the potency of the serum on June 14 is attributable to an insufficiency of antigen in the preceding dose (see above).

#### *Horse 2.*

The immunisation of a second horse was begun on June 18, 1917. During the first 18 days the horse received seven increasing doses of killed spirochaetes contained either in liver emulsions or in culture fluids.

A sample of serum taken five days after the seventh injection and 23 days after the beginning of the immunisation was tested for immune bodies.

#### *First potency test.*

Number of guinea-pig	Quantity of serum	Duration of life of guinea-pigs	Result
2 controls	—	Both dead, 4 days	Typical spirochaetosis
2469	1 c.c.	Died, 18 days	No haemorrhages or jaundice
2470	0.5 c.c.	Died, 15 days	Typical spirochaetosis
2471	0.25 c.c.	Died, 11 days	do.

This test showed that 1 c.c. of the serum was capable of protecting a guinea-pig from a dose of spirochaetes which killed controls in four days while smaller amounts of the serum prolonged the lives of the guinea-pigs.

The immunisation of the horse was continued with living spirochaetes derived either from cultures or from the livers of fatally infected guinea-pigs.

A second test of the serum was made after the sixth dose of living virus, 54 days from the beginning of immunisation.

*Second potency test.*

2 controls	—	Both dead, 4 days	Typical spirochaetosis
2482	0.5 c.c.	Died, 31 days	No sign of disease
2483	0.25 c.c.	Survived	
2484	0.125 c.c.	do.	

Another guinea-pig receiving 1 c.c. of the serum two days after the test dose of emulsion also survived.

This test showed that the serum had at least eight times the potency of the first sample.

As the doses increased in size it was found more convenient to use as principal antigen liver emulsion than culture. Liver emulsions contain enormous numbers of spirochaetes and to obtain approximately equal numbers in cultures would have required very considerable quantities of nutrient media. Whenever cultures were available however these were administered along with the emulsions. The largest dose of culture injected at one time was 30 cubic centimetres.

On September 18, eight days after the nineteenth dose of antigen and three months from the beginning of immunisation, the horse was bled 6 litres.

*Third potency test.*

Three sets of experiments were carried out with this serum. In the first two the smallest quantity of serum used was 0.05 c.c. and this in the two instances in which it was given afforded complete protection from a dose of spirochaetes which killed controls in from six to nine days.

A third series was done in order to ascertain the minimum quantity of serum which would protect, and the result is set out in the following table:

3 controls	—	All dead, 4 days	Typical spirochaetosis
2543	0.05 c.c.	Died, 104 days	No disease
2544	0.025 c.c.	Survived	—
2545	0.0125 c.c.	Died, 9 days	Typical spirochaetosis

On November 24, eight days after the 25th dose, which was 66 c.c. of an emulsion made from the livers of five guinea-pigs, the horse was bled 8 litres.

*Fourth potency test.*

2 controls	—	Died in 5 days	Typical spirochaetosis
2580	0.0125 c.c.	Survived	
2581	0.01 c.c.	Died, 6 days	No jaundice or haemorrhages
2582	0.01 c.c.*	Died, 5 days	
2583	0.006 c.c.*	Died, 6 days	No jaundice, slight haemorrhages
2584	0.005 c.c.*	Died, 6 days	Typical spirochaetosis

\* Incubated with test dose 45 minutes.

This test showed that since September 18 the serum had increased considerably in potency. 0.0125 c.c. now protected a guinea-pig completely from a dose which was fatal to controls in five days, while 0.01 c.c. was able apparently to destroy the spirochaetes in the test dose, though unable to save the lives of the guinea-pigs (microscopical examination of the liver of one of these latter guinea-pigs failed to reveal a spirochaete).

The immunisation of the horse was continued until the middle of March 1918, by which time 33 immunising doses had been given. On March 28 the animal was bled again. The potency of this batch of serum has not yet been tested because of the loss of the strain of spirochaetes which was being passed through guinea-pigs. Since January 1917 the strain had been passed from guinea-pig to guinea-pig until March 1918 when owing to a temporary shortage of young guinea-pigs adult guinea-pigs had to be used. The first of these died in seven days of spirochaetosis, but material from it failed to produce the disease in a second adult guinea-pig.

The first batch of serum sent out for the treatment of cases was from the first horse taken before the last and fatal dose of antigen. The titre of this serum was not very high (between 0.2 and 0.1 c.c.) but it was higher than that of the serum (titre 0.5 c.c.) used by the Japanese in their first series of cases. It was therefore supplied for trial in any case which might arise before a more potent serum became available.

The therapeutic results obtained by the Japanese workers (Inada, Ido, Hoki, Ito and Wani, 1916) with the low titred serum were very favourable. The serum considerably reduced the mortality rate and was found to be capable of destroying completely the spirochaetes in the circulating blood. Moreover the treatment promoted the development of antibodies and reduced the number of spirochaetes in the organs. At first they injected 10 c.c. of the serum into the subcutaneous tissues daily for three days but experience showed that this dosage was ineffectual. They therefore increased it gradually until finally they injected 60 c.c. in 24 hours. Later on they injected the serum intravenously and they found that this method far exceeded in potency subcutaneous injection. In a recent publication (1918) these observers give the results of the intravenous serotherapy of 41 cases of infectious jaundice. The spirochaetocidal titre of the sera used in this series was 0.01 and 0.03 c.c.

As a rule they injected 60 c.c. intravenously irrespective of severity of illness. Sometimes the entire quantity was given within 24 hours; at other times 40 c.c. in one day and 20 c.c. on the following day, or 20 c.c. on three successive days.

The action of the immune serum is spirochaetocidal and spirochaetolytic and best results are obtained when injections are made at an early stage of the disease. They found that intravenous injections are effective up to the fifth and sixth days of illness.

The total mortality figures, particularly of the severer cases, was considerably lower in the serum treated cases (intravenous and subcutaneous) than in the non-serum treated cases.

When the serum is administered early the disease appears to assume a milder form. The immune serum shortens the duration of the illness and has also a definitely beneficial influence upon haemorrhages, heart rhythm and suppurative processes.

The results obtained in this series led the authors to the conclusion that a titre of 0.01 c.c. suffices for the efficient serotherapy of infectious jaundice.

The November 24 serum from my second horse attained to this standard and should therefore be as effective in the treatment of the disease as the Japanese serum.

I have not yet any results to record of the therapeutic use of the serum.

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## THE ATYPICAL DYSENTERY BACILLI.

BY T. J. MACKIE, M.B., D.P.H.,

*Professor of Bacteriology, University of Cape Town.*

As there appears to be a considerable amount of uncertainty (as evidenced by recent writings on this subject) regarding the biological relationships and the etiological significance of the so-called "atypical *B. dysenteriae*," this short communication is intended as a brief résumé of conclusions arrived at in the course of an extended investigation of dysentery bacilli in Egypt during 1916 and 1917. It is to be noted that the results recorded here were obtained from the examination of cases of dysentery observed in the earliest stages and frequently followed into convalescence. Such experience has shown that conclusions derived solely from an investigation of the later stages of the disease, *e.g.* convalescents examined after arrival from over-seas in the United Kingdom, throw little light on the problem of the bacterial etiology of the disease as a whole. In a paper by Thomson and Mackie (1917)<sup>1</sup> on the clinical and laboratory study of dysentery in Egypt a number of atypical dysentery strains were described, but at the time this communication was submitted for publication my observations were not sufficiently complete to make any general statements regarding organisms of this type and no attempt was made to classify them.

When atypical and inagglutinable strains were first encountered in the course of laboratory examination of dysentery cases, the tendency was to disregard their occurrence and only the classical types were accepted for diagnostic purposes. As time went on, however, and as the proportion of cases from which classical dysentery bacilli could be isolated was unexpectedly low, attention was once more directed to these atypical varieties especially when they occurred in large numbers in the excreta during the earlier phase of an acute case in which amoebae were absent. Some of them corresponded in all their cultural reactions to the Shiga or Flexner-Y types, but failed to react to specific agglutinating sera. These came to be designated "inagglutinable *B. dysenteriae* Shiga or Flexner-Y." Only a few of these became agglutinable after repeated subcultures on artificial media, when tested two to three months after isolation, and some which in primary culture appeared to correspond to this designation rapidly underwent spontaneous mutation

<sup>1</sup> *Journ. Royal Army Med. Corps*, xxviii. 403. The present author is personally responsible for the bacteriological notes in this paper.

and displayed fermentative characters which clearly differentiated them from the classical types. The close biological similarity to the group of classical dysentery bacilli was nevertheless very striking, and from their constant occurrence in a certain proportion of all the acute dysenteries and their characteristic toxic effects on animals (*v. infra*), they came to be accepted as dysentery producing organisms.

#### CLINICAL TYPES OF DYSENTERIC INFECTIONS.

Dysenteric infections displayed a considerable diversity in their manifestations and varied from the typical acute attack of dysentery with the characteristic blood and mucus stools, to a simple diarrhoeal illness without obvious blood or mucus discharges. Generally speaking the latter type was characterised by the presence of more or less abundant cellular exudate evident on microscopic examination of the stools. Thus two types of infection might be distinguished: (1) an acute type with blood and mucus in the stools, (2) a milder type with fluid stools containing abundant cellular exudate but without blood or mucus. This distinction is of importance, as will be seen later, in determining the relationship of the atypical dysentery bacilli to the classical organisms as regards their pathogenic effects. The typical dysentery bacilli (Shiga and Flexner-Y types) were found to be associated with both the severe and the milder infections, but the great majority of the Shiga infections were of the acute type while, in the case of the Flexner-Y infections, the proportion of acute cases was smaller and the number of ordinary diarrhoeal cases larger as compared with the Shiga infections (see also below).

#### *B. DYSENTERIAE* SHIGA STRAINS.

The Shiga strains isolated invariably corresponded in their cultural and biochemical reactions to the classical type and showed a specific agglutination reaction (*i.e.* to end titre) with a Shiga agglutinating serum. Only a few strains which corresponded in cultural reactions to the Shiga type and did not agglutinate in primary culture, became agglutinable after repeated subculture. No non-mannite fermenting strains, which resembled the Shiga type in most of their cultural characters, but differed as regards the fermentation of maltose or the production of indol, ever agglutinated with the Shiga serum. I originally thought that such strains and also the so-called inagglutinable *B. Shiga* might be "variants" from the classical type, and this was noted in the original paper by Thomson and myself. I ultimately classified these along with the atypical dysentery bacilli.

#### *B. DYSENTERIAE* FLEXNER-Y STRAINS.

In the ultimate identification of organisms of the Flexner-Y type, the Lister Institute Y serum<sup>1</sup> was used: and it may be said that the strains of

<sup>1</sup> Prepared with the original strain of Hiss and Russell, see Chick, *Lancet*, April 22, 1916.

this group invariably corresponded in their cultural characters to the classical types and showed a *specific* agglutination reaction with this serum to end titre. Saccharose fermenting strains which reacted to specific serum have been described by other observers, *e.g.* Martin<sup>1</sup>, Glynn and others<sup>2</sup>, but I invariably found that the Flexner-Y strains failed to ferment saccharose and lactose, and that mannite fermenting strains which after some days' incubation fermented saccharose or lactose were not agglutinated by the Y serum, accordingly they were classified with the atypical group.

A "Flexner Serum" (R.A.M. College) was also used in parallel series in the agglutination tests of a considerable number of mannite fermenters and only a small proportion of these reacted to it even in low titres.

I prepared a high titre agglutinating serum to a strain which reacted specifically to the Lister Y serum, but not to the Flexner serum, and found that this serum only agglutinated a small number of strains which were agglutinated to end titre by the Lister Y serum, and none of these reacted to the Flexner serum. This may be represented as follows:

	Lister Y serum	Flexner R.A.M. College serum	Serum to a strain agglutinated by Y serum
A. Majority of strains of Flexner-Y group ... ..	+	-	-
B. A small number of strains of Flexner-Y group ... ..	+	+	-
C. A small number of strains of Flexner-Y group (not the same strains as are included in group B)	+	-	+

(+) indicates agglutination up to "end titre" of the serum.

This subject requires further investigation, but it would appear that the Flexner-Y group includes perhaps a number of species and that the strain with which the Lister Y serum is prepared represents antigenic properties common to practically the whole group. A strain which was agglutinated by the Flexner serum was invariably agglutinated by the Y serum. Chick<sup>3</sup> has also noted the more restricted degree of specificity shown by "Flexner" sera for mannite fermenting strains as compared with the action of Y serum. It is further noteworthy that some strains of "Shiga" are agglutinated up to end titre by this "Y" serum. Only a small number of strains found to be inagglutinable in primary culture became agglutinable after subculture.

With regard to the agglutination of organisms of the dysentery group, it is to be noted, that, as contrasted with the typhoid group, sedimentation occurs comparatively slowly and the clumps are much smaller and the sediment is granular rather than flocculent. The results recorded here were obtained by incubating organisms (suspensions of 24 hour agar-cultures) and serum for two hours at 37° C. and then allowing the tubes to stand overnight at room-temperature.

<sup>1</sup> Martin, *Brit. Med. Journ.* i. April 14, 1917.

<sup>2</sup> Glynn, Berridge, Foley, Price and Robinson, *Report to Medical Research Committee*, December, 1917.

<sup>3</sup> *Lancet*, April 22, 1916.



THE "ATYPICAL *B. DYSENTERIAE*."

This group may now be defined as (1) Gram-negative, non-motile bacilli, not liquefying gelatin, always fermenting glucose without gas production, (2) different strains varying as regards the fermentation of lactose, dulcitol, mannite, maltose (but never producing gas in any case) and the formation of indol from peptone, and (3) not agglutinated by a Y, Flexner, or Shiga serum. The cultural reactions when once acquired are all stable, as determined by repeated examination of strains. The reactions of some of the types met with are shown in Table I.

Table I.

*Atypical Dysentery Bacilli.*

Atypical <i>B. dysenteriae</i>	Motility	Glucose	Lactose	Dulcitol	Saccharose	Mannite	Maltose	Indol	Gelatin	
No. 1	-	↓	-	-	-	-	-	-	-	{ Corresponds to <i>B. dysenteriae</i> Shiga but not agglutinable by specific anti-Shiga serum
2	-	↓	-	-	-	-	-	+	-	
3	-	↓	-	-	-	-	↓	-	-	
4	-	↓	-	-	-	-	↓	+	-	
5	-	↓	-	-	-	↓	-	-	-	{ Correspond to <i>B. dysenteriae</i> Flexner-Y but not agglutinable by specific anti-Y serum
6	-	↓	-	-	-	↓	-	↓	-	
7	-	↓	-	-	-	↓	↓	-	-	
8	-	↓	-	-	-	↓	↓	↓	-	
12	-	↓	-	-	↓	-	↓	+	-	
16	-	↓	-	-	↓	↓	↓	+	-	
17	-	↓	-	↓	-	-	-	-	-	
22	-	↓	-	↓	-	↓	-	+	-	
24	-	↓	-	↓	-	↓	↓	↓	-	
32	-	↓	-	↓	↓	↓	↓	+	-	{ Corresponds to <i>B. dysenteriae</i> Strong
40	-	↓	↓	-	-	↓	↓	+	-	
48	-	↓	↓	-	↓	↓	↓	+	-	
56	-	↓	↓	↓	-	↓	↓	+	-	
64	-	↓	↓	↓	↓	↓	↓	+	-	

↓ - acid no gas.

These reactions when once acquired are all stable as determined by repeated examination of strains.

The numerical classification is based on the various possible combinations of cultural reactions.

All the various types were proved to be extremely virulent by intravenous or intraperitoneal injection of rabbits, producing a characteristic haemorrhagic enteritis, which was most marked in or limited to the small intestine. In fact, the virulence experiments demonstrated a highly selective toxic action on the mucosa of the small intestine (affecting the stomach to a less degree), which on autopsy was found to be intensely inflamed with massive haemorrhages in the tissue, and the lumen of the intestine was usually distended with blood-stained muco-purulent material loaded with cellular exudate and

masses of exfoliated epithelium. In the case of intraperitoneal injections, often there was little reaction in the peritoneum, but the characteristic effect on the intestine was distinct. Cultures from the intestinal contents usually yielded an almost pure growth of the particular organism. These results were obtained as a rule by injecting  $\frac{1}{8}$  or  $\frac{1}{4}$  of an agar slope culture in saline and the animals died in about 24 hours. Some strains, however, including a number of atypical varieties, exhibited a much higher virulence and the intravenous injection of  $\frac{1}{40}$  of a 24 hours' agar slope culture produced the characteristic haemorrhagic enteritis. Comparative tests with a recently isolated Shiga and a No. 2 strain (see table) showed that the latter was distinctly the more virulent. This capacity for producing haemorrhagic enteritis may be regarded practically as an attribute peculiar to the dysentery group—typical and atypical. The lesion differs from the punctiform haemorrhages sometimes met with in the intestinal wall in various types of septicæmia.

From their occurrence in large numbers in early cases of acute dysentery, in the absence of the classical types, and their characteristic effects in animal experiments (which were found to be similar to those produced with the classical Shiga and Flexner-Y types), together with their close biological similarity to organisms of the typical dysentery group, it was concluded that these atypical organisms were to be regarded as true dysentery bacilli<sup>1</sup>. This group, of course, includes organisms which have been designated "inagglutinable Shiga and Flexner types," but it would appear more rational to avoid this designation and class all the organisms with the group characters given above as atypical or paradysentery bacilli. In this group mutations and also late fermentations were frequently noted, and organisms which would on first examination have been designated inagglutinable Flexner varieties soon developed additional fermentative characters.

#### CLINICAL SIGNIFICANCE OF ATYPICAL *B. DYSENTERIAE*.

As regards the type of infection due to these organisms, the majority of the cases were of the milder type and the proportion of cases with the typical acute signs, *i.e.* passing of blood and mucus, was lower. Nevertheless severe types of dysentery were not infrequently met with apparently due to these varieties. To sum up, the Shiga infections were mostly of the severe type and the atypical *B. dysenteriae* infections of the milder type, while the Flexner-Y infections occupied an intermediate position in this respect. Atypical organisms have been isolated from stools within a few minutes from the time they were passed, and were the predominating organism present. Thus there is no support for the suggestion that these are accidental contaminations in faeces kept for some time.

<sup>1</sup> In the case of infections with *B. Shiga* and *B. Flexner* the agglutination reaction of the patient's serum was found to be so variable and unreliable and so frequently absent, except in such low titres (1:50 or less) as to introduce the fallacy of a normal serum effect, that no investigation of this reaction was carried out in the case of the atypical infections.

## MIXED INFECTIONS.

In certain instances mixed infections with typical and atypical organisms were noted, but these were relatively uncommon.

METHOD OF ISOLATING *B. DYSENTERIAE*.

It is not out of place here to refer briefly to the isolation of the dysentery bacilli. The medium I have generally employed and found most satisfactory has been MacConkey's agar. The use of a modified MacConkey's medium containing trypsinised heart extract did not appear to give appreciably better results. On MacConkey's medium the colonies of the Shiga bacillus were usually the smallest, and those of the atypical organisms the largest, but considerable variations were noted. The faeces must be examined as soon as possible after evacuation; repeated cultures from numerous specimens have shown that in general at room-temperature (Egypt in winter) the dysentery organisms cease to be recoverable after six to eight hours, although exceptionally they may persist much longer.

## FREQUENCY OF DYSENTERY BACILLI IN THE FAECES AT DIFFERENT STAGES OF THE DISEASE.

During the first few days of the illness, the dysentery bacilli were present in enormous numbers and often in almost pure culture; after this they tended to disappear and to be replaced by "concomitant organisms," see Table II, *e.g.* *B. Morgan* No. 1 and other similar organisms (*B.C.L.A.*, Nos. 1, 2, 3, etc.),

Table II.

*Concomitant Bacilli.*

				Motility	Glucose	Lactose	Dulcitate	Saccharose	Mannite	Malto-e	Indol	Gelatin
<i>B. Morgan</i> No. 1	...	...	...	+	+	-	-	-	-	-	+	-
<i>B.C.L.A.</i> No. 1	...	...	...	+	+	-	-	-	-	-	-	-
„ No. 2	...	...	...	-	+	-	-	-	-	+	+	-
„ No. 3	...	...	...	-	+	-	-	-	-	-	+	-
„ No. 6	...	...	...	+	+	-	-	-	-	+	+	-
„ No. 7	...	...	...	-	+	-	-	-	-	-	-	-
<i>B. faecalis alkaligenes</i>	...	...	...	+	-	-	-	-	-	-	-	-
<i>B. paracolon</i> types	...	...	...	+	+	-	+	-	+	+	+	-
<i>B. proteus</i> types	...	...	...	+	+	-	-	+	-	+	+	+

All gram-negative bacilli.

The cocco-bacillus referred to in the text is morphologically coccal, but with only a few bacillary forms.

+ = acid and gas in the fermentation tests.

*B. faecalis alkaligenes*, *B. paracolon* types, *B. proteus*, *B. pyocyaneus*, *Staphylococci*, and a Gram-negative non-motile non-carbohydrate-fermenting coccobacillus. Thus at a later stage of a dysenteric illness, plate cultures showed large numbers of colonies of these concomitant bacilli and dysentery bacilli were absent. It is, of course, difficult to determine the actual part played by these organisms, but it would appear as if the dysentery bacilli proper often only initiated the lesions and that these other organisms acted in aggravating or maintaining the disease. In a considerable number of autopsies at various stages in which cultures were made directly from the floor of intestinal ulcers and from necrotic tissue no dysentery bacilli were isolated, but cultures of these various concomitants were obtained. These results render the significance of bacteriological findings in convalescent cases of extremely dubious value so far as throwing light on the etiology of bacillary dysentery is concerned.

## THE ASSOCIATION OF *RICKETTSIA* WITH TRENCH FEVER.

BY J. A. ARKWRIGHT, M.D., A. BACOT AND F. MARTIN DUNCAN.

(*From the Lister Institute of Preventive Medicine.*)

(With Plates II and III.)

THE following contains a preliminary account of the work on the Etiology and Pathology of Trench Fever which has been done at the Lister Institute in connection with the War Office Committee on Trench Fever under the Chairmanship of Major-General Sir David Bruce, F.R.S. A first account from the Clinical side has been published in a paper by Major Byam, R.A.M.C., and his colleagues at the New End Military Hospital, Hampstead, and read before the Society for Tropical Medicine and Hygiene in May of last year. We have been mainly dependent for our material on Major Byam and the rest of the Medical Staff of the New End Hospital and are very much indebted to them for their help and courtesy. We are under an especial obligation to Lieut. L. Lloyd, R.A.M.C., who has carried out that part of the entomological work done at the Hospital, taking immense pains in supervision and in obtaining the material which we required.

### HISTORY AND DESCRIPTION OF *RICKETTSIA* BODIES.

The class of microorganism to which the name of *Rickettsia* has been given by da Rocha-Lima is associated chiefly with two human diseases, Typhus and Trench Fever and the lice which transmit them. The parasites found in Rocky Mountain Spotted Fever are probably very closely related; they are described as occurring in blood films and in very large numbers in the Tick, which transmits this disease, by Ricketts (1909), Wolbach (1916, 1918). A fourth species has been found by Nöller (1917) in the "sheep tick," *Melophagus ovinus*; this species, *R. melophagi*, is not known to be associated with any mammalian disease. Töpfer (i. 1917) further associates war nephritis with another form of *Rickettsia*.

The chief characters distinctive of *Rickettsia* are the following: (1) morphology: they are of very small size,  $0.3$  to  $0.5 \times 1.5$  to  $2.0$  microns. Their shape resembles a coccus, diplococcus or a short bacillus. They stain rather feebly by aniline dyes, do not retain Gram's stain, and are not acid fast, but stain well by Giemsa, when they appear as small dots, double cocci, or bipolar staining bacilli with an unstained central part. They are non-motile. (2)

Occurrence in the blood: they occur in blood films sparsely, and are best seen in dehaemoglobinised thick drops taken during the periods of fever (Rocky Mountain Fever, Ricketts, 1909; Typhus, Ricketts and Wilder, 1910; Trench Fever, Töpfer, x. 1916). It is, however, now generally acknowledged that the recognition of scanty *Rickettsia* in the blood or tissues is very difficult and uncertain. (3) Occurrence in the insect vector: Ricketts and Wilder (1910) found these microorganisms in the louse in Mexican Typhus. Sergeant, Foley and Viallatti (1914), da Rocha-Lima (1916), Töpfer and others confirmed this observation and described enormous numbers of the parasite in the midgut of lice that had bitten a patient during the height of the fever a few days previously. (4) Artificial culture: attempts to cultivate *Rickettsia* on artificial media have been usually unsuccessful, but Nöller records that he grew *R. melophagi* on a blood agar medium. The claims made by Töpfer (1917) and Csernel (1916) appear to have been based only on single occurrences and are unconfirmed.

There has been a tendency to regard *Rickettsia* as a Protozoon as first suggested by Prowazek and favoured by da Rocha-Lima. The reasons for this view appear to be largely *a priori*, on account of the fact that it is insect-borne, the relapsing character of the fever in Trench Fever, and in addition the peculiar staining properties attributed to the bodies by da Rocha-Lima. However Ricketts, Wilder, Töpfer and the present writers find that with Giemsa the staining reaction is very like that of other bacteria.

Nevertheless, this class of microorganism and its associated diseases appear to have sufficiently distinct characteristics to justify the retention of the name *Rickettsia* for the present.

#### THE ASSOCIATION OF TRENCH FEVER AND *RICKETTSIA* BODIES.

The presence of the virus of Trench Fever in the blood of patients during and just after a febrile attack was shown by McNee. Renshaw and Brunt (12. ii. 1916) by transmitting the disease to man by intravenous or intramuscular injection of blood. This has been confirmed and amplified by the War Office Committee on Trench Fever in England, and the American Red Cross Medical Research Committee in France (1918); the latter also made additional experiments on plasma, filtered material, etc. A few inoculation experiments in Germany (Werner, Benzler and Wiese (ix. 1916)) have also been published. Several observers (Jungmann (iii. 1916), Töpfer (iii. 1916) and others) claim to have seen definite bodies in the blood in wet and dry preparations which resemble diplococci or bipolar staining bacilli and are like those described by Ricketts in Rocky Mountain Spotted Fever, and by Ricketts and Wilder in Typhus.

The suspicion that lice were concerned in the spread of Trench Fever was suggested in McNee's paper, and has been supported by the evidence of Davies and Weldon (3. ii. 1917). Jungmann and Kuczinski (iii. 1917), Werner and Benzler (v. 1917) also make statements on the subject, which, however, do not afford very satisfactory proof of this mode of transmission.

The War Office Committee working in England (Byam (v. 1918)) and the American Medical Research Committee in France have shown conclusively that the disease is readily transmitted by lice from patients to healthy volunteers. The former Committee has shown that the excreta or the body contents of infected lice, rubbed into a scarified area on the skin of a healthy man almost invariably reproduces the disease, usually with relapses, and other characteristic symptoms after an incubation in man of six to 14 days. They also showed that infected lice fed for over 30 days on several healthy men failed to infect them, although the lice were frequently fed on Trench Fever patients during that time. The latter Committee found that when infected lice were allowed to feed on healthy men the majority contracted Trench Fever after a period of about 2-4 weeks had elapsed.

Töpfer (17. x. 1916) described microorganisms in lice from Trench Fever patients which very closely resembled those found in lice from Typhus Fever, but claimed that he could distinguish the two kinds of parasites—those associated with Trench Fever being shorter and thicker than those from Typhus lice, but he did not consider important da Rocha-Lima's distinction between Typhus and other kinds of *Rickettsia*, namely, the invasion of the epithelial cells of the gut wall by the former. He states that some of the lice caught on every Trench Fever patient contained these parasites, and that uninfected lice fed on Trench Fever patients were found subsequently to harbour similar forms. These microorganisms were first found in the midgut of lice on the 5th day after the first infecting feed and were present in very large numbers on and after the 8th day. Not every louse was found to be infected.

Jungmann and Kuczinski (23. iii. 1917) state that they regularly produced a fatal infection in wild mice with the blood of Trench Fever patients injected intraperitoneally and that they found the same bipolar staining parasites in the peripheral blood of the mouse and in the patients' blood. They also, like Töpfer, found that the guts of lice, which had fed on patients, became infected with parasites, but they could not distinguish them from those found in Typhus lice. They say that in both diseases the microorganism develops inside the epithelial cells of the louse's midgut. They state that the gut of an infected louse injected intraperitoneally into a mouse causes its death in the same way as when a patient's blood is inoculated. They infected normal lice by letting them bite Trench Fever patients, and both these and also 80 % of the lice caught on Trench Fever patients were found to be infected on microscopical examination.

Munk and da Rocha-Lima (30. x. 1917) confirm the occurrence and multiplication of *Rickettsia* in lice fed on Trench Fever patients. Da Rocha-Lima claims that parasites found in lice fed on patients suffering from the two diseases can be distinguished by the position in and destruction of the insects' gut-cells by *Rickettsia prowazeki* which is associated with Typhus, and the irregular and only occasional entrance into the cells by *Rickettsia*

*quintana* and *R. pediculi* which occur in lice from Trench Fever cases and normal lice respectively. He acknowledges that he is unable to distinguish *R. quintana* from *R. pediculi*. He maintains that the inability of other workers to distinguish *R. prowazeki* from the other two species is due to their examination having been made by means of smears, whereas a differentiation can only be made with certainty by examining serial sections 3-5 microns thick, of which he claims to have examined over 25,000. He also describes slight morphological differences.

#### ANIMAL EXPERIMENTS.

Da Rocha-Lima states that he infected guinea-pigs and produced a characteristic temperature chart by inoculating various kinds of material (blood, urine, lice) from patients. However, only seven out of 44 animals reacted typically, ten slightly and 27 not at all. He was unable to pass the disease on to other guinea-pigs from those first infected. He could not infect mice. He records 119 experiments made by feeding normal lice on 103 persons suffering from Trench Fever, or other fevers, or who were normal. He used lice from places which were believed to be free from Typhus, or lice bred in captivity. Lice which showed doubtful appearances or only scanty *Rickettsia* were excluded from the results. They were examined partly by smears and partly by serial sections. Of 70 experiments on Trench Fever patients 51 gave a positive result, i.e. at least one louse was found to contain *Rickettsia*. In 11 the lice remained negative. In 33 control experiments on men not suffering from Trench Fever, but who were in hospital with other diseases or were healthy, the lice fed on 26 gave negative results and those from six positive results; the lice from one of these latter were heavily infected though the patient had never had any general symptoms.

Of 14 persons examined in Hamburg, where there had been no Typhus fever, in two the majority of the lice were strongly infected. One of these was a man who had been associated with soldiers from the front.

Trench Fever patients were able to infect lice with *Rickettsia* both before, during and after a febrile attack, and after many weeks of convalescence.

#### TIME OF APPEARANCE OF *RICKETTSIA* IN THE LOUSE.

After the first experimental infecting feed on a patient, *R. quintana* was commonly found in lice on the 3rd to the 6th day, sometimes in large numbers on the 4th day. Lice caught on patients showed the parasites usually on the 6th to the 8th day. *R. prowazeki* was commonly well seen in lice after the 8th day from the first experimental feed on Typhus patients, and only exceptionally as early as the 4th or 5th day.

Trench Fever lice remained healthy, but Typhus lice often died. Lice infected with *R. quintana* remained infected after long continued feeding on a healthy man.

Da Rocha-Lima failed to infect by the bites of lice containing many



*Rickettsia* of the *R. pediculi* type. Korbsch (1916) reports a failure after two "infected" lice had fed on him for eight days, and also no result following scarification and rubbing in of a single "infected" louse on two occasions.

Strisower (iv. 1918) described three cases of transmission of Trench Fever to men by feeding infected lice on them and also claims to have infected mice and cats in series. Several other writers (e.g. His and Stintzing) criticise the work on *Rickettsia* adversely and support the hypothesis of a spirochaete as the infective agent.

#### DISCUSSION OF THE FURTHER EVIDENCE IN THE LITERATURE AS TO THE CAUSAL RELATION BETWEEN TRENCH FEVER AND *RICKETTSIA*.

(1) The hypothesis that *Rickettsia* causes Trench Fever is largely founded on the analogy of Typhus Fever in which the evidence brought forward is more complete owing to experiments on monkeys and guinea-pigs. The claims to have infected guinea-pigs, cats, mice and rabbits with Trench Fever in a recognisable form are very unconvincing in the published reports.

(2) Observations on forms resembling *Rickettsia* in the blood of Trench Fever patients are very difficult to interpret and unsatisfactory, since these forms are so scanty and their morphology alone when present in small numbers in a film is inconclusive.

(3) The inability to obtain growth of *Rickettsia* on artificial culture media make inoculation and re-infection experiments, after several subcultures, impossible at present.

(4) The chief reasons for the failure to obtain acceptance or a better hearing for *Rickettsia* as the probable cause of Typhus and Trench Fever are: (a) The not very rare occurrence according to Continental observers of *Rickettsia* in lice from sources where Typhus and Trench Fever have not been suspected; (b) connected with this objection, and perhaps largely the cause of it, is the difficulty in distinguishing the forms of *Rickettsia* associated with Typhus and Trench Fever from each other, and from those found in "normal" lice. There is no agreement as to definite means of distinguishing these parasites, but da Rocha-Lima's claim to differentiate them by their position in serial sections is more convincing than Töpfer's very slight description of differences in morphology. (c) The confusion has perhaps been enhanced by the failure of the German observers to work with a clean stock of lice. Da Rocha-Lima did use some lice bred in the laboratory, but he makes no statement as to their number or the proportion of such lice used or the results obtained with them, as distinguished from lice caught in surroundings believed to be free from infection. Moreover, the captive lice would require feeding on some human being and the infection of laboratory workers with Trench Fever is common.

The wide distribution and high incidence of Trench Fever on the Continent during the War makes it very difficult to obtain definitely uninfected lice for experiments or uninfected men on whom to feed them, except by breeding

lice, feeding them on a man of unimpeachable freedom from Trench Fever infection, and watching their excreta through several generations.

(5) The counter-claims of other workers to have discovered the cause of Trench Fever in a spirochaete mainly rest on the very insecure basis of occasional observations of a single spirochaete in the blood, supported by *a priori* arguments, except in the case of (1) Riemer (i. 1917) who, in addition, obtained these organisms in culture from one patient, but his observations have been unconfirmed, and (2) Couvy, Dujarric and de la Rivière (12. i. 1918), who experimentally infected guinea-pigs with patients' blood and passed the infection on to other guinea-pigs; these observers recovered the spirochaetes in considerable numbers from the kidneys, etc. Their observations have so far not been corroborated by other workers.

#### THE AUTHORS' OBSERVATIONS AND EXPERIMENTS.

Early in 1918 it had become clear from the experimental work on volunteers of the Committee (Byam 1918) that the virus of Trench Fever was contained in a very active form in the excreta and bodies of lice, since the body contents of 11 lice or a small pinch of dry excreta was sufficient to infect a man through the excoriated skin.

Much microscopic and cultural work had been done on the blood of patients by previous workers, at the New End Hospital, Hampstead, especially by Captain Dimond, and, since its formation, by the members of the War Office Committee.

It was determined therefore early in May, 1918, to concentrate our attention on the excreta and contents of the intestinal canal of infected lice, while some cultural and other observations on the blood of patients were continued.

Cultures of the excreta and guts of lice, both infected and normal, had sometimes yielded a variety of bacteria and sometimes been sterile, but one form of *Bacillus* was the most constant and had been previously studied by Bacot. In young cultures on agar or blood agar it takes the form of a coccus 1.5 to 2.0 microns in diameter and is often seen dividing. In older cultures, and sometimes even in the first 24 hours, films show irregular threads amongst the round or oval organisms. When first isolated growth takes place best at about 27° C., but later it grows well at 37° C. It is Gram-negative and non-motile; ferments glucose, mannite and lactose very slowly; forms acid and clot in milk in about 14 days, and is not pathogenic for guinea-pigs. Attempts were then made by means of wet and dry films and by cultures to discover other organisms in the excreta of infected lice which were not present in those of normal lice.

One or two films made by the late Professor Plimmer from the excreta of infected lice and fixed wet with formalin and iodine vapour showed an immense number of very small particles which were probably minute organisms, and turned our attention to a search for the *Rickettsia* described by Töpfer and Rocha-Lima. In many films of excreta, whether fixed wet or dry, a large

number of stained granules were seen, which recalled descriptions of *Rickettsia*, but it was very difficult to distinguish the granules of altered blood, etc., from the more definite forms, and one was continually in doubt as to whether one was looking at minute microorganisms or precipitated protein. In order to get rid of the débris of red corpuscles, the films after drying were fixed for one or more hours in absolute alcohol containing 20 drops of strong hydrochloric acid per c.c. as recommended for thick blood drops in Malaria work by W. M. James. By using this technique, there has seldom been difficulty in distinguishing *Rickettsia* when present from granules of débris, though the disintegrating nuclei of leucocytes occasionally have presented a somewhat similar appearance. The chief further source of error lies in the danger of confusing these parasites with other microorganisms, especially larger bacteria only stained in the centre or at the poles, and with the small bacteria occasionally seen, which approach *Rickettsia* in size.

The criteria which we have adopted in deciding on the presence of *Rickettsia* have been (1) its minute size, smaller than *M. melitensis* or *B. influenzae*, usually about  $0.3 \times 0.3$ , or  $0.3 \times 0.5 \mu$ ; (2) its irregularity in shape, round, oval, diplococcal or bacillary with stained poles; (3) its occurrence in very large numbers, or even in masses, especially on flakes of solid material in the excreta; (4) its well-stained appearance when coloured by Giemsa, the colour being purple like that of the nucleus of a leucocyte.

When small granules, somewhat resembling *Rickettsia* in size and shape, but stained pink or lilac, have been met with, or if the bodies have been very few and scattered about the film, the result of microscopical examination has been recorded as negative or doubtful. The number of "doubtful" specimens has very much diminished as our experience has increased, and now it is very seldom necessary to return an uncertain result, though *Rickettsia* no doubt are sometimes present but unrecognized on account of their rarity in the film.

#### METHOD OF CONDUCTING EXPERIMENTS WITH LICE.

A large, healthy stock of *Pediculus corporis*, maintained by Bacot for over three years, was used. The lice were confined in boxes, covered with fine gauze, and were fed twice daily by the method described by him (Bacot, 1917). When not in use the lice were kept in an incubator or in an inside pocket at  $27^{\circ}$  to  $30^{\circ}$  C. except in those experiments in which another temperature is recorded. The lice in each box usually numbered 50 to 100. They were fed once or twice for 20 to 30 minutes on Trench Fever patients during an attack of fever, also in some instances during non-febrile periods; thereafter they were fed on a healthy man or in some experiments on the same infected man throughout.

Excreta were examined by shaking them as a dry powder out of the boxes through the gauze, and making an emulsion on a glass slide with a drop of salt solution. The contents of the gut were examined by dissecting it out

and emulsifying it on a slide with needles in a small drop of salt solution. The film was dried and fixed in acid alcohol and stained with Giemsa. Dead lice were also examined by soaking the body in a drop of salt solution and teasing it up on a slide. It was found that films made from dead lice showed *Rickettsia* very clearly when these were present.

Some infected and uninfected lice were also examined by serial sections.

#### INCUBATION OF *RICKETTSIA* IN THE LOUSE.

It was found that if a boxful of lice were given an infecting feed the excreta obtained from the box did not show *Rickettsia* for some days.

Table I.

Showing the results of examining microscopically the excreta of lice on a series of days after the first infecting feed, and also the results of inoculation of excreta from two of the boxes, 150 and 134.

Days from first infecting feed	Box No.											
									150		134	
	175	142	A 15	A 18	A 25	A 34	193	A 33*	Microsc. exam.	Result of inoculation	Microsc. exam.	Result of inoculation
1st	—	—	...	—	—	—	...	—	—	—	—	—
2nd	...	+ ?	...	—	—	—	...	—	...	...	...	...
3rd	...	—	...	—	—	—	—	—	...	...	— ?	—
4th	+	—	...	...	—	—	—	—	...	...	...	...
5th	+ ?	—	—	—	—	...	—	...	+	+	— ?	—
6th	—	—	...	+ ?	—	—	—	—	—	—	...	...
7th	...	+	...	—	—	—	—	—	++	+	...	...
8th	...	+	+++	+++	—	—	—	—	—	—	+	—
9th	+	+	++	...	—	+	—	—	+	+	...	...
10th	++	+	...	+++	+++	+	—	—	+	+	...	...
11th	...	+	+++	+++	+++	+	+	—	+	+	...	...
12th	++	+	...	+++	+++	+	+	—	...	...	...	+
13th	...	+++	+++	+++	+++	+++	++	—	...	...	+	...
14th	...	+++	...	+++	...	++	+++	...	...	...	...	...
15th	...	...	...	+++	...	++	++	—	...	...	...	...
16th	++	+++	+++	+++	...	...	+++	—	...	...	...	...
17th	...	++	...	+++	+++	+++	++	—	...	...	...	...
18th	...	++	...	+++	...	...	...	...	...	...	...	...
19th	...	+	...	+++	+++	...	...	...	...	...	...	...
20th	+++	+	...	+++	+++	...	+++	...	...	...	...	...
21st	...	...	...	+++	+++	...	...	...	...	...	...	...
22nd	...	...	...	+++	...	...	...	...	...	...	...	...
23rd	...	...	...	...	...	...	...	...	...	...	...	+
24th	...	...	...	...	...	...	...	...	...	...	+	...

+ = *Rickettsia* seen in a few microscope fields.

++ = " " several " "

+++ = " " enormous numbers.

— = No *Rickettsia* seen.

... = No examination.

\* The lice in boxes A 33 and A 34 were fed at the same time, but A 33 was kept at about 20° and A 34 at 27° C.

When *Rickettsia* have once been found in a box in large numbers they usually continue to be found in daily examinations till all the lice of the infected generation are dead.

Table I shows the results of a daily microscopical examination of the excreta from ten of the boxes of experimental lice which had previously fed on a Trench Fever patient. The results of a series of experimental inoculations into volunteers of the excreta from two of the same boxes, 150 and 134, are also shown. It is seen, (1) that *Rickettsia* appear in the excreta after a series of negative examinations following the infecting feed, and (2) that the numbers of these bodies can often be seen to be smaller when they first appear than on later days, (3) also that when the infection is thoroughly established a positive result is obtained every day. The period elapsing between the first infecting feed and the recognition of *Rickettsia* varies from about 4 to 10 days in this series. Examinations of boxes kept at about 20° C. remained consistently negative; though the lice were apparently healthy.

The results shown in this table, *i.e.* an incubation period, the appearance of *Rickettsia* after about a week or ten days, and its persistence for three or four weeks, have been a constant phenomenon when we have been able to examine a series of specimens.

Table II relates to experiments with 20 boxes of lice which were fed on ten different patients with Trench Fever. The boxes were examined at frequent intervals, in most cases daily, either by making films from the excreta, or from lice. It is seen that *Rickettsia* was first seen on the 5th to the 12th day, most commonly on the 7th to the 10th day from the first infecting feed, when the lice were kept at about 27° C. between the feeds. Boxes A 20 b, A 28, A 30, and A 33 were kept at about 20° C. and *Rickettsia* did not appear, though examinations were continued till the 17th, 19th and 22nd day from the infecting feed in these experiments. It was also shown that if a box was kept at 20° C. for two or three days and then at 27° C. the parasites were found after a week at the higher temperature.

Lice in eight other boxes fed on seven infected patients (six of whom were different men from those mentioned in Table II) have been found to be infected with *Rickettsia* after a variable number of days, but the examinations have not been sufficiently numerous to determine the date when these first appeared. In two of them, however, the parasites were present in the excreta on the 6th day and in one on the 7th day from the first infecting feed.

Seven other boxes of lice, which have been fed on patients believed to be suffering from Trench Fever, have only been examined on two or three occasions, and *Rickettsia* has not been definitely found.

Table II also shows the number of days which elapsed after an infecting feed before the excreta were proved to be infective, *i.e.* capable of producing Trench Fever when inoculated into volunteers in two series of experiments.

In the case of Box 134 the excreta from the 1st to the 8th day did not infect, but those collected on the 12th day reproduced the disease, whereas

*Rickettsia* were first demonstrated on the 8th day. The excreta from Box 150 showed *Rickettsia* on the 5th day and the same specimen of excreta infected a volunteer. Excreta from Box A 18 showed the parasite microscopically on the 8th day, and a mixed sample of excreta collected on the 8th to the 22nd day proved virulent for man, but they were not tested earlier for virulence.

Table II.

Showing the day after the first infecting feed on which *Rickettsia* was first found in the guts or excreta of lice.

In the case of Boxes 134 and 150 the day when infective excreta were first obtained is also shown.

No. of boxes of lice	Source of infection of lice			Material examined	Day from infecting feed on which	
	Ref. No. of patient	Day of disease	Febrile or non-febrile		Microscopic result first positive	Successful inoculation obtained
162	{ Ex. 46 Ex. 21	2nd 11th	Febrile Non-febrile	Midgut	10th	...
175	Ex. 60	1st	Febrile		9th	...
134	G.	79th	"	Excreta	8th	12th*
150	Ex. 33	2nd	"	"	5th	5th*
142	Ex. 27	2nd	"	"	7th	...
A 15	A.	2nd	"	"	8th	...
A 16	A.	5th	Non-febrile	"	5th	...
A 18	A.	10th	Febrile	"	8th	...
A 20 a†	A.	23rd	Non-febrile	"	10th	...
A 24	Ex. 73	25th	Slightly febrile	"	9th	...
A 25	C.	43rd	Non-febrile	"	10th	...
A 27	A.	29th	"	"	12th	...
A 29‡	Ex. 81	19th	Febrile	"	9th	...
A 31§	Ex. 81	30th	Slightly febrile	"	7th	...
A 34	A.	49th	Non-febrile	"	9th	...
A 35	A.	49th	"	"	7th	...
A 20 b†	A.	23rd	"	"	Negative 19th	...
A 28‡	Ex. 81	19th	Febrile	"	" 17th	...
A 33	A.	49th	Non-febrile	"	" 17th	...
A 30§	Ex. 81	30th	Slightly febrile	"	" 22nd	...

\* See also Table I.

† Boxes A 20 a and A 20 b were alike and treated in the same way except that A 20 a was kept at 27° C and A 20 b at 20° C.

‡ Boxes A 28 and A 29 were alike and treated in the same way except that A 28 was kept at 20° C. and A 29 at 30° C.

§ Boxes A 30 and A 31 were alike and treated in the same way except that A 30 was kept at 20° C. and A 31 at 27° C.

|| Boxes A 33, A 34, A 35 were alike and treated in the same way except that A 33 was kept at 20° C., A 34 at 27° C. and A 35 at 32° C.

These facts show a general agreement in that the virus of Trench Fever and *Rickettsia* both require an incubation period of 4 to 12 or more days in the louse before they are demonstrable—the former by inoculation, the latter microscopically. This point will be dealt with in more detail further on.

The lice in Boxes 162, 175, 134, 150 and 142 were fed on the infected man

for one day only, and afterwards on a healthy man. Those in the remaining boxes in Table II were fed twice daily on the same or another infected man. The day of the disease in the patient on whom the lice were fed, and whether he was febrile or non-febrile at the time of the first infecting feed, are also shown.

Lice may be infected on a Trench Fever patient when he is febrile or between the attacks, or even some weeks after the last fever has been noticed, as in the case of Boxes 163 and A 16, which were fed in the intervals between attacks, and Boxes A 20 a, A 27, A 34 and A 33, which were fed on the 22nd, 28th, 48th and 48th day of the disease, and the 13th, 19th, 39th and 39th days respectively since any fever had been observed, or any symptoms complained of.

#### *RICKETTSIA* NOT TRANSMITTED BY HEREDITY IN LICE.

The offspring of infective lice fed on a normal man have not shown, with one exception, *Rickettsia* either in their excreta or in their stomach contents.

On 15 June, 1918, nits were taken from Box 163, the excreta from which were known to contain the virus of Trench Fever and numerous *Rickettsia*. The nits were divided into two batches, I and II.

*Batch I.* These were put into a clean box without being treated.

*Batch II.* These were put for three minutes into 1 % lysol and then washed in a current of tap water for several minutes, dried and put in another clean box. Both boxes were kept subsequently at about 30° C. and as soon as larvae were hatched out they were fed twice daily on a healthy man.

On 15 July the excreta from Box I were examined and one small flake crowded with *Rickettsia* was found, but no other parts of the film showed these forms. Three subsequent examinations of the excreta from Box I and three examinations of the excreta from Box II all gave negative results. It seems clear that the flake with *Rickettsia* was a portion of excreta carried over on the unwashed eggs.

When newly hatched larvae, descendants of the infected lice in Boxes 15 and 16, were fed twice daily on an infected man their excreta contained the parasites on the 8th and 5th days respectively, and not before.

Table III.

Showing the proportion of lice found on dissection to be infected with *Rickettsia* at different periods.

	Day after first infecting feed								
	0-7th day			8th-14th day			15th onwards		
	+	-	Total	+	-	Total	+	-	Total
Series 1	3	24	27	7	5	12	15	0	15
" 2	0	3	3	5	4	9	43	17	60
Both series together	3	27	30	12	9	21	58	17	75

+ = *Rickettsia* found.

- = No *Rickettsia* recognized.

In Series 1 comprising lice from six boxes the lice only fed on an infected man during 24 hours, and afterwards on a healthy man. In Series 2 (eight boxes) the lice fed on an infected man from the first infecting feed onwards. When individual lice from a recently infected box are dissected and examined it is found that only a few are infected with *Rickettsia* in the first week after the first infecting feed, that during the second week about an equal number are infected and uninfected, and that after the second week the majority show parasites microscopically.

Table III gives the actual figures in a series of lice taken at different stages from 14 boxes of infected lice, and examined microscopically. The increase in the number of infected lice is very marked.

In microscopic sections of infected lice *Rickettsia* were seen crowding the region of the epithelial cells lining the alimentary canal, but there was no definite invasion of the cells. Appearances like those figured by da Rocha-Lima from sections of Typhus lice in which the epithelial cells show well defined areas which are badly stained and occupied by masses of *Rickettsia* were not seen.

#### EXAMINATION OF NORMAL LICE.

For comparison with these boxes of lice which have been infected by feeding on Trench Fever patients a number of boxes of uninfected lice have also been examined frequently, some daily for weeks, by making films of the excreta. Also a considerable number of normal lice have been dissected and films from the midgut examined, and serial sections have been cut of others. Only in one box on one occasion have forms closely resembling *Rickettsia* been found. Seeing how difficult or impossible it is to distinguish bacteria by their morphology, an occasional error is not surprising. When these organisms occur in an infected box they are almost always found on many successive days and not only on one single occasion, as was the case with the apparently exceptional occurrence among normal lice mentioned above.

In all 22 boxes of lice fed on normal persons have been examined repeatedly over periods lasting usually over two months and never less than 14 days. These normal persons on whom they have been fed are seven in number, and two stocks of normal lice from different sources are under observation.

Two other boxes of lice supposed at the time to be normal, besides the one referred to above, showed *Rickettsia*. They were both being fed on A. who had been also feeding infected lice for over five weeks and had been working daily with infected excreta in the laboratory. He developed Trench Fever on June 11th. The first box, A 12 a, showed *Rickettsia* in the excreta on June 8th, and of nine lice dissected on June 10th three gave a positive result, the other box, A 10, showed infected excreta on June 14th. It does not seem reasonable to include these findings in Boxes A 10 and A 12 a as positive results from normal lice.

Some other lice found on healthy civilians have been examined but none have been found to be infected with *Rickettsia*.



The total number of specimens of lice or excreta from boxes of lice which had been fed on men believed to be infected with Trench Fever was 253, and of these 150 showed *Rickettsia*, 83 gave a negative and 20 a doubtful result. Of these specimens of excreta collected during the first week after the first infective feed, 14 were positive and 73 were negative.

Second week, 75 were positive and 27 were negative.

Third week, 61 were positive and 3 were negative.

Of 245 specimens from 22 boxes of normal lice fed on seven healthy persons, only one was positive (if the four specimens from A's two boxes mentioned above are excluded), 234 gave a negative and 10 a doubtful result.

#### CORRELATION OF THE PRESENCE OF *RICKETTSIA* AND TRENCH FEVER VIRUS, IN THE LOUSE.

The association of *Rickettsia* and infectivity for man of the lice containing them is very striking; *Rickettsia* and the virus of Trench Fever also have certain properties in common.

1. The size of the *Rickettsia* is such that one would expect them to be held back by a good Berkefeld filter; they nevertheless approach the lower limits of size of known bacteria.

In an experiment by McNee with filtered and unfiltered blood plasma, the unfiltered alone transmitted the disease. The American Medical Research Committee (1918) in France have been able to transmit the disease by means of filtered material. They have also found that plasma, freed from cells by centrifuging, is still virulent. In experiments with emulsion of louse excreta in salt solution, we have found that only prolonged centrifuging at high speed (2500–3000 revolutions) for 20–30 minutes will produce a definite deposit of *Rickettsia*. They may therefore be separable from blood corpuscles by fractional centrifuging in blood plasma also, but we have been unable by centrifuging to demonstrate their presence in more than small numbers in citrated plasma of Trench Fever patients diluted 1 in 5.

2. The blood in Trench Fever is infective by direct inoculation during a febrile attack, and, at any rate sometimes, when there is no fever. Lice can also be infected with Trench Fever virus over a long period, whether the patient is febrile or not; they also become infected with *Rickettsia* when fed on patients during similar periods, e.g. the excreta of lice (Box 163) fed on the patient St. when non-febrile on the 27th to 28th day of the disease gave Trench Fever to several volunteers and contained large numbers of *Rickettsia*; the lice in Box 134 fed on another patient G. on the 79th day of his disease, gave Trench Fever to a volunteer by means of excreta collected on the 12th day from the infecting feed. The excreta from this box first showed *Rickettsia* on the 8th day.

3. Lice fed on patients are able to transmit the disease if their excreta or midgut contents are rubbed into scratches or inoculated subcutaneously. They, however, appear not to become infective (two series of experiments)

till the 5th to the 12th day after the infecting feed. In the same way *Rickettsia* is not in our experience recognizable in lice or their excreta when kept at 27° C. till the 5th to the 12th day (usually the 7th to the 10th day) after the first infecting feed on a patient.

4. In an infected box not every louse appears to contain the virus of Trench Fever as tested by inoculation of man, neither does every louse contain *Rickettsia* in recognizable form or amount. Only 12 out of 21 examined in the second week from the infecting feed showed these parasites. In one experiment two lice were selected from an infected box (A 16 a) on about the 13th day after the first infecting feed. The midguts were dissected out and emulsified separately. It was shown by microscopic examinations of both the gut contents and the excreta of these lice that the one, A, contained many *Rickettsia* and the other, B, did not. Inoculation of the emulsions separately into two volunteers by scarification, produced Trench Fever in the one inoculated with A and not in the one inoculated with B.

5. A box once infected appears to remain so for two or three weeks, both as regards the virus of Trench Fever and the presence of *Rickettsia*, i.e. during the life-time of the infected generation of lice.

6. The high infectivity of louse excreta is associated with the presence of enormous numbers of *Rickettsia*, whereas the difficulty of finding these bodies in blood films is well known.

7. Lice or the excreta of lice which have been proved to contain virus, as the result of successful inoculation, have in most cases, when examined, been shown to contain large numbers of *Rickettsia*. Certain exceptions to this have occurred. It is however not surprising that a small sample of excreta should occasionally fail to show the parasite which may be present in large numbers in another part of the same excreta. On the other hand, if uniformly distributed, *Rickettsia* might escape recognition though present in considerable numbers.

In Table IV are shown all the experiments made by inoculating volunteers with the excreta or body contents of lice in which the specimen has also been examined microscopically. The result of such examination is shown in column 3 and of the inoculation experiment in column 5. The 6th column states whether there is agreement (A.), disagreement (Dis.), a doubtful result (?) or a reason for agreement not being expected (O.).

Fifty-six specimens are included in Table IV. Fifty-three were samples from boxes of lice which had had a feed on an infected patient, of these 50 were samples of excreta and three were emulsions of single lice. Three further specimens of excreta are included in the Table—two from boxes of normal lice fed on healthy men and both examined microscopically and inoculated into volunteers, as control experiments; the third specimen is from a box containing the offspring of infected lice which were examined to test the hypothesis of hereditary transmission of the virus of Trench Fever or of *Rickettsia*. Out of the 53 specimens from boxes of lice which had been

Table IV.

Showing the results of microscopical examination and inoculation of 54 specimens of louse excreta and of three single lice.

1 No. of Box of Lice	2 Source of infecting feed		3 <i>Rickettsia</i> in specimen	4 Days from infecting feed	5 Inoculation of man		6 Agree- ment or dis- agree- ment	7 Incuba- tion in man. Day	Notes
	Name	Date			No. of Ex- peri- ment	Date			
Mixed	...	...	+++	...	10	9. ii.	+	A.	6th
Mixed	...	...	+++	...	11	9. ii.	+	A.	7th
Mixed	...	...	+++	...	12	9. ii.	+	A.	9th
Mixed	...	...	+++	...	20	5. iii.	+	A.	9th
Mixed	...	...	+++	...	21	16. iii.	+	A.	8th
134	G.	20. iii.	-	1st	23	21. iii	-	A.	...
134	G.	20. iii.	-	3rd	24	23. iii.	-	A.	...
134	G.	20. iii.	-	4-5th	25	25. iii.	-	A.	...
134	G.	20. iii.	+	* 6-8th	26†	28. iii.	-	Dis.	...
134	G.	20. iii.	{ + }	12th	27	1. iv.	{ + }	A.	8th
				13th					
Mixed	...	...	+++	...	28	28. iii.	-	O.	... Immunity
Mixed	...	...	+++	...	29	30. iii.	-	O.	... Immunity
134	G.	20. iii.	{ + }	23rd	30	13. iv.	{ + }	A.	7th
				24th					
Mixed	...	...	+++	...	32	6. iv.	+	A.	8th Excreta untreated
Mixed	...	...	+++	...	33	6. iv.	+	A.	8th Excreta heated 56° C.‡
Mixed	...	...	+++	...	34	6. iv.	-	O.	... Excreta heated 80° C.§
150	J.	15. iv.	-	1st	39	17. iv.	-	A.	...
150	J.	15. iv.	-	6th	41	21. iv.	-	A.	...
150	J.	15. iv.	++	7th	42	22. iv.	+	A.	13th
150	J.	15. iv.	-	8th	43	23. iv.	-	A.	...
150	J.	15. iv.	+	9th	45	26. iv.	+	A.	9th
150	J.	15. iv.	+	10th	46	26. iv.	+	A.	7th
Mixed	...	...	+	...	51	7. v.	+	A.	8th
150	J.	15. iv.	+	5th	53	2. v.	+	A.	16th Few <i>Rickettsia</i> ; none seen in 2nd film
150	J.	15. iv.	-	6th	{ 41 54 }	2. v.	{ - }	A.	...
						2. v.			
150	J.	15. iv.	++	7th	55	2. v.	+	A.	13th
150	J.	15. iv.	+	9-10-11th	58	10. v.	+	A.	12th
150	J.	15. iv.	+	11th	59	2. v.	+	A.	8th
163	S.	7. v.	+++	11-12-13th	64	20. v.	+	A.	7th
163	S.	7. v.	+++	21-22-23rd	70	1. vi.	+	A.	7th
157	W.	23. iv.	+	? 26-31st	71	31. v.	+	?	8th One louse
155 } 164 }	T.	...	-	11-24th	72	1. vi.	+	Dis.	10th
163	S.	7. v.	+++	11-14th	73	2. vi.	+	A.	9th
163	S.	7. v.	+++	11-24th	74	5. vi.	-	O.	... Excreta treated with lysol 2 %

A. = results agree.

Dis. = results disagree.

O. = results not comparable.

\* A very few *Rickettsia* were found in only one out of three films.

† This man was inoculated three times with negative result (Exps. 43 and 63).

‡ Dry heat for 20 mins.

§ Moist heat for 10 mins.

Table IV—continued.

Showing the results of microscopical examination and inoculation of 54 specimens of louse excreta and of three single lice.

1 No. of Box of Lice	2 Source of infecting feed		3 <i>Rickettsia</i> in specimen	4 Days from infecting feed	5 Inoculation of man			6 Agree- ment or dis- agree- ment	7 Incuba- tion in man. Day	Notes
	Name	Date			No. of Ex- peri- ment	Date	Result			
163	S.	7. v.	+++	11-24th	75	8. vi.	+	A.	10th	
170	C.	14. v.	+	12-20th	76	9. vi.	-	O.	...	Immunity
163	S.	7. v.	+++	11-13th	77	11-13. vi.	+	A.	11th	
163	S.	7. v.	+++	11-24th	80	14. vi.	+	?	12th	Influenza (?)
163	S.	7. v.	+++	11-24th	81	14. vi.	+	A.	10th	
163	S.	7. v.	+++	14-36th	82	14. vi.	-	O.	...	Excreta heated to 70° C. 20 mins.—moist
171	B.	15. v.	+++	11-19th	85	16. vi.	-	O.	...	48th day of disease— immunity
177	P.	29. v.	-	12-20th	86	18. vi.	+	Dis.	9th	
173	T.	17-18-19. v.	±	14-17th	90	19. vi.	-	?	...	Immunity
150	J.	15. iv.	-	8th	91	19. vi.	-	A.	...	
155 } 164 }	T.	...	-	11-24th	92	22. vi.	-	A.	...	
173	T.	17-18-19. v.	±	14-17th	93	22. vi.	-	?	...	
163	S.	7. v.	+++	11-36th	94	2. vii.	-	O.	...	Excreta heated to 99.9° C. 20 mins.—dry
A 18	A.	20. vi.	+++	8-22nd	96	19. vii.	+	A.	7th	2 mg. excreta sub-cut.
A 18	A.	20. vi.	+++	8-22nd	97	19. vii.	+	A.	11th	0.1 mg. excreta sub-cut.
Mixed	R.*	...	+++	...	98	23. vii.	-	O.	...	Immunity—excreta kept 154 days
171	B.	15-18. v.	+++	11-19th	99	23. vii.	+	A.	9th	
A 16 a	A.	13. vii.	-	11th	100	24. vii.	-	A.	...	One louse
A 16 a	A.	13. vii.	+++	11th	101	24. vii.	+	A.	9th	One louse
122	Healthy man		-	...	22	18. iii.	-	A.	...	Offspring of infected lice
127-9	Healthy man		-	...	17	12. iii.	-	A.	...	Lice were fed on a heal- thy man as a control experiment
127-9	Healthy man		-	...	18	12. iii.	-	A.	...	

A. = results agree.

Dis. = results disagree.

O = results not comparable.

fed on Trench Fever patients, three gave uncertain results from microscopic examination and in one the reaction following inoculation was of a doubtful nature, as the attack of fever was attributed to influenza, nine are excluded from the results because agreement between the results of the two methods of examination was not to be expected, either on account of the disinfection of the excreta by lysol or heat, or because the volunteer had previously gone through an attack of Trench Fever and was probably immune—the inoculation being in fact given as a test for immunity.

Of the 40 remaining, 27 gave positive results, both as regards the presence of *Rickettsia* and the virulence of the samples, 10 gave negative results from both tests, and three different results from the two tests.

There was therefore agreement as to the presence or absence of *Rickettsia* and virus in 37 out of the 40 samples which gave a decided answer, *i.e.*

92.3 % of agreement. In one of the three samples in which there was disagreement the microscopic examination was positive, and the virulence test negative; in the other two the reverse was the case.

The specimens of excreta from which results showing agreement were obtained were in some instances inoculated into more than one man. There are eight such observations recorded which were repetitions of former experiments. In seven of these the two results were positive and in one negative. In every case the excreta when used for a second experiment gave the same result as on the first occasion. Excluding the repetitions the total number of experiments is 32 of which 29 showed agreement (90.6 %) and three disagreement.

8. There is evidence that the virus of Trench Fever is not inherited in the louse, since excreta of the offspring of infected lice fed on healthy men from the egg have failed on inoculation to produce Trench Fever; the excreta of the offspring of the infected lice in several boxes have on examination not shown *Rickettsia*. One apparent exception to this statement occurred (see above).

#### CONCLUSIONS.

1. The intimate association in lice of *Rickettsia* with the virus of Trench Fever appears to have been amply proved.

2. The examinations of lice which have fed on healthy civilians in England have given negative results in a sufficiently uniform manner to constitute a significant negative control, but further examinations of lice from normal civilians are desirable.

3. Lice from soldiers who have been in France, or who have mixed with men from France in this country, would not afford a satisfactory control, since the infection of Trench Fever with the power of infecting lice with Trench Fever virus and with *Rickettsia* may be very long lasting.

4. Whether *Rickettsia* constitute the virus of Trench Fever or are in some way produced by it remains undecided because *Rickettsia* cannot be cultivated on artificial media.

5. It is conceivable that *Rickettsia* are not living microorganisms, but their appearance certainly suggests that they are bacteria, and their remarkable association with Trench Fever virus in the louse further suggests that they are the causal agent of Trench Fever.

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## DESCRIPTION OF PLATES II AND III.

- Figs. 1 and 3. Films of excreta of infected lice from Box A 16, made 30. vi. 18 on the 15th day from the first infecting feed on a Trench Fever patient.
- Fig. 2. Film of excreta of infected lice from Box A 18, made 30. vi. 18 on the 10th day from the first infecting feed. Stained Giemsa.  $\times 1000$ . Showing pure "culture" of *Rickettsia*.
- Figs. 4 and 5. Films made from the fore-gut of an infected louse from Box 171, made 10. vi. 18 on the 26th day from the first infecting feed. Showing numerous *Rickettsia*—pure.
- Fig. 6. Film of excreta of normal lice fed on healthy man; showing bacteria which are sometimes found in the excreta of normal lice. Stained Giemsa.  $\times 1000$ .
- Figs. 7 and 8. Sections of the hind-gut of an infected louse from Box 160, killed 4. v. 18, the 7th day after the first infecting feed. Stained Giemsa.  $\times 1000$ . Showing *Rickettsia* on the surface of the epithelium. *a*, body-cavity. *b*, gut-wall. *c*, lumen of gut containing altered blood, and, close to the epithelial cells, *Rickettsia*.



Fig. 1



Fig. 2



Fig. 3







Fig. 4



Fig. 5



Fig. 6

*a*

*a*



Fig. 7

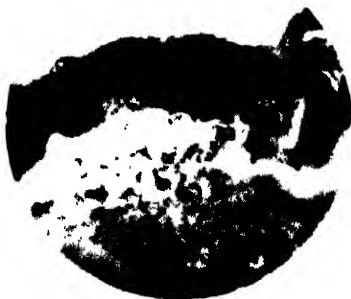


Fig. 8



# THE INFLUENCE OF THE AGE OF PARENT AT BIRTH OF OFFSPRING UPON THE DEVELOPMENT OF EYE COLOUR AND INTELLIGENCE—A CORRECTION.

By R. J. EWART, M.D., D.Sc.

OWING to some regrettable slips in the arithmetical work of the paper dealing with eye colour and intelligence recently published in this *Journal*<sup>1</sup> several of the constants are inaccurate. The whole of the calculations have now been re-worked and the correct values are given below. I have incorporated some fresh material, the analysis of which somewhat strengthens the conclusions already published.

I shall first discuss the new material. Table I records the eye colour distributions of infants aged from one to six months by categories of parental age and the resultant correlation. Although the estimated probable error, derived by the formula appropriate to product moment correlations, is not a complete measure of the random fluctuations of such coefficients as these, the relation found would appear to be of some significance and is actually the largest yet obtained from material of this class.

An obvious criticism is that the age distribution of the infants may be materially different for different arrays of parents. To test the importance of this, an extended series of observations was made upon children, from birth

Table I.

*Influence of Age of Parent at Birth and Eye Colour of Young Infant (one to six months of age). (Barking.)*

Age of Parent	Eye colour. Infant.			Percentage not blue
	Blue	Not blue	Total	
16th to 20th years	16	0	16	0
21st „ 24th „	59	6	65	9.2
25th „ 28th „	65	13	78	16.1
29th „ 32nd „	55	12	67	17.9
33rd „ 36th „	34	7	41	17.1
37th „ 40th „	35	12	47	25.5
41st „ 44th „	10	2	12	16.6
Totals	274	52	326	

$$r = +.206 \pm .036.$$

<sup>1</sup> *Journ. of Hygiene*, xvi. 1917, pp. 12-35.

*Influence of the Age of Parent*

to the age of one year, the material being derived from an infant clinic (Tables II-IV). The resulting constants are:

Standard Deviation, Mother's Age	...	...	...	1.5894 ± .0386
" " Child's "	...	...	...	2.9905 ± .0726
Correlation of Child's Eye Colour and Mother's Age	...	...	...	.115 ± .035
" " " " Child's "	...	...	...	.348 ± .030
" " Age " Mother's "	...	...	...	.034 ± .035

Hence the partial correlation of mother's age and child's eye colour. Child's age constant

$$r = .110 \pm .034.$$

Table II.

*Child's Age and Mother's Age.*

Child's age in weeks	Mother's age in years								Totals
	16-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	
1-4	3	11	11	8	5	5	2	—	45
5-8	7	23	20	26	15	14	5	1	111
9-12	3	12	15	8	9	11	1	—	59
13-16	2	7	10	7	4	6	2	—	38
17-20	2	1	14	8	4	3	1	—	33
21-24	—	6	6	6	4	3	—	—	25
25-28	1	5	4	2	3	2	2	—	19
29-32	1	7	5	2	3	3	—	—	21
33-36	—	—	—	—	1	3	—	1	5
37-40	—	2	3	1	2	—	—	—	8
41-44	—	—	2	1	—	—	1	—	4
45-48	—	2	1	4	2	—	—	—	9
49-52	1	1	1	2	2	2	—	—	9
	20	77	92	75	54	52	14	2	386

Table III.

*Child's Age and Eye Colour.*

Child's age in weeks	Child's eye colour		
	Blue	Not blue	Total
1-4	43	2	45
5-8	103	8	111
9-12	51	8	59
13-16	24	14	38
17-20	23	10	33
21-24	21	4	25
25-28	14	5	19
29-32	19	2	21
33-36	2	3	5
37-40	4	4	8
41-44	3	1	4
45-48	4	5	9
49-52	7	2	9
	318	68	386

Table IV.

*Child's Eye Colour and Mother's Age.*

Age of mother in years	Child's eye colour		
	Blue	Not blue	Total
16-20	20	—	20
21-24	66	11	77
25-28	74	18	92
29-32	60	15	75
33-36	45	9	54
37-40	39	13	52
41-44	12	2	14
45 and over	2	—	2
	318	68	386

It appears therefore that some weight must be assigned to the objection offered but that when it is allowed for correlation persists.

In Table V the age of the father is taken into consideration. Information on this point being obtained indirectly through the mother, it is doubtless not very accurate. The correlation, of the same order of magnitude as found for the mother and child, rather suggests that any biological significance attaching to the previous results is not dependent upon intra-uterine nutritive changes.

Table V.

*Age of Father at Birth of Child. (Children under 1 year when observed.)*

Father's age in years	Children's eye colour		
	Blue	Not blue	Total
16-20	1	—	1
21-24	29	6	35
25-28	64	12	76
29-32	67	12	79
33-36	37	11	48
37-40	42	13	55
41-44	19	5	24
45-48	15	4	19
49-52	4	1	5
53-56	1	1	2
57-60	2	—	2
	281	65	346

Standard Deviation, father's age, 1.849.

Coefficient of correlation,  $r = .0924 \pm .036$ .

I now pass to the correction of previously published results. The numbering of the tables is that of the paper cited.

*Middlesbrough School Children (younger).*

	Boys	Girls
Tables VIII and VII. Age of parent at birth and eye colour ...	$r = .138 \pm .032$	$.048 \pm .032$
„ X and IX. Age of parent at birth and child's eye colour ...	$r = .149 \pm .032$	$.071 \pm .032$
„ XI and XII. Eye colour, mother and child ...	$r = .524 \pm .024$	$.623 \pm .019$

## *Influence of the Age of Parent*

### *Making the Third Factor Constant in Each Case.*

	Boys	Girls
Tables VIII and VII.	$r = .070 \pm .033$	$.046 \pm .032$
„ X and IX.	$r = .092 \pm .032$	$.053 \pm .032$
„ XI and XII.	$r = .514 \pm .020$	$.622 \pm .019$

### *Barking School Children (older).*

Table II. Eye colour, mother and child...	...	...	...	...	...	$r = .608 \pm .020$
„ III. Age of parent at birth and child's eye colour	...	...	...	...	...	$r = .005 \pm .032$
„ IV. Age of parent at birth and her own eye colour	...	...	...	...	...	$r = .009 \pm .034$

### *Making the Third Factor Constant in Each Case.*

Table II.	$r = .607 \pm .020$
„ III.	$r = .001 \pm .034$
„ IV.	$r = .008 \pm .032$

### *Random Observation.*

Table V. Eye colour of any mother with any child but its own	...	...	...	...	...	$r = .030 \pm .072$
„ V. Age of parent at birth with eye colour of any child but its own	...	...	...	...	...	$r = -.068 \pm .026$

### *Adult Life.*

Table XIII. Age of grandmother at birth of mother and mother's eye colour	...	...	...	...	...	$r = .003 \pm .022$
Table XIV. Represents Tables VII and VIII taken together and refers to mother's eye colour only, that is, "Age at birth and her own eye colour"	...	...	...	...	...	$r = .093 \pm .022$

Taking the chief constants we have:

1. Young infants	...	...	...	...	$r = .206 \pm .036$
2. Young school children, boys	...	...	...	...	$r = .092 \pm .032$
„ „ „ girls	...	...	...	...	$r = .052 \pm .032$
3. Older school children	...	...	...	...	$r = .001 \pm .034$
4. Adults	...	...	...	...	$r = .003 \pm .022$

From these figures it is seen that the value of  $r$  obtained in samples of a young infant population disappears or becomes very small in sampling older children. In the younger school children the correlation for girls is not significant, for boys it may be so and therefore may indicate some bias. Hence I suggest that the rate of change of eye colour is more rapid in the later born, and that of the two sexes, the boys are probably somewhat later than the girls in reaching the full development of pigmentation.

If the development of shade, from the primitive blue of birth, is studied, it would seem that none of the ultimate colours can be regarded as transitional stages, hence if a scale could be devised, blue must occupy the middle position, the brown being towards one end and the grey towards the other. Or rather the primitive blue in the centre and the resultant colours occupying some position on the surface of a sphere. Hence there may be some justification for the division of eye colour into three groups, grey, blue and brown, and for treating them as though the distribution were Gaussian.

If this assumption is justifiable, then we may arrange our data in the following way, taking first the question as to whether there is a significant

difference in distribution of eye colour in those women who reproduce before and after the 30th year.

*Reproducing Women.*

Age	Brown	Blue	Grey, etc.	Totals
40 years and under at time of observation	246 (33·5)	234 (31·9)	253 (34·5)	733
41 years and over at time of observation... ..	181 (30·7)	161 (27·3)	247 (41·9)	589
	427	395	500	1322

30 years and under at time of birth, 40 years and under at time of examination.

Distance of brown from  $\bar{x}$  = ·4245  
 „ „ grey „  $\bar{x}$  = ·3988  
 ·8233

Standard Deviation 1·214 ± ·019.

31 years and over at time of birth, 41 years and over at time of examination.

Distance of brown from  $\bar{x}$  = ·5046  
 „ „ grey „  $\bar{x}$  = ·2035  
 ·7081

Standard Deviation 1·412 ± ·023.

From this it is seen that there is a slight and perhaps significant difference between the two groups, dependent upon a diminution in the number classed as blue and a large increase amongst the greys. But it must be remembered that the range of age in the latter class is from 41 years to 58 years, and the former from 28 years to 40 years, and that some change is to be expected on that account.

It seems reasonable therefore to suppose that so far as these observations allow, there is no definite evidence of selection with respect to eye colour in a population of reproducing women.

Turning to the school children:

*School Children. All ages from 7 years.*

*Boys and Girls. Eye Colour.*

	Brown	Blue	Grey, etc.	Totals
Born at age of 30 years and under	357 (28·4)	582 (46·3)	318 (25·3)	1257
„ „ 31 „ „ over	269 (31·1)	340 (39·3)	256 (29·6)	865
	626	922	574	2122

30 years and under.

Distance of brown from  $\bar{x}$  = ·5715  
 „ grey „  $\bar{x}$  = ·6653  
 1·2368

Standard Deviation ·809 ± ·013.

31 years and over.

Distance of brown from  $\bar{x}$  = ·4934  
 „ grey „  $\bar{x}$  = ·5361  
 1·0295

Standard Deviation ·9713 ± ·020.



At least *prima facie*, the distribution deviates from the parental type and in the opposite direction so far as the relation of age to the proportion classed as brown is concerned. I do not, however, desire to put much weight upon constants deduced on an assumption which is somewhat arbitrary.

It has occurred to me that the existence of correlation when parental age is correlated with the eye colours of young children and its evanescence when older children are involved may be a reflection of a phenomenon suggested by earlier results, viz. that the variability of filial arrays increases with the parental age at birth. Given a surface of zero regression but with increasing array variability, truncations of it should exhibit correlation. This may be illustrated in the special case of Gaussian arrays.

Suppose that all  $x$  arrays of  $y$  are Gaussian and further that every  $\bar{y}_x = \bar{y}$ . Then the correlations of the two surfaces formed by dividing the original surface by a plane intersecting the axis of  $y$  at right angles in the line  $y = 0$  are equal and opposite if  $\sigma_{y_x}$  increases with  $x$ .

Let  $\bar{x}_1, \sigma_{x_1}, \bar{x}_2, \sigma_{x_2}, \bar{y}_1, \sigma_{y_1}, \bar{y}_2, \sigma_{y_2}$  be the means and standard deviations of the two halves, all measurements being from the means.

Then

$$x_1 = \bar{x}_2 = \bar{x} = 0,$$

$$\sigma_{x_1} = \sigma_{x_2} = \sigma_x,$$

$$\bar{y}_1 = -\bar{y}_2,$$

$$\sigma_{y_1} = \sigma_{y_2}.$$

Consider the contribution made to the sum product  $S_1 xy$  by the array  $y_{x=-s}$ . It is

$$-\frac{s\alpha_{-s}}{\sqrt{2\pi}\sigma_{y_{x=-s}}} \int_{-\infty}^0 ye^{-\frac{y^2}{2\sigma_{y_{x=-s}}^2}} dy = \frac{s\alpha_{-s}\sigma_{y_{x=-s}}}{\sqrt{2\pi}},$$

where  $\alpha_{-s}$  is a function of  $x = -s$ .

Similarly the contribution of the array  $y_{x=+s}$  is

$$-\frac{s\alpha_{+s}\sigma_{y_{x=+s}}}{\sqrt{2\pi}}.$$

Thus the contribution of corresponding arrays is

$$\frac{s}{\sqrt{2\pi}} (\alpha_{-s}\sigma_{y_{x=-s}} - \alpha_{+s}\sigma_{y_{x=+s}}),$$

and the complete product

$$\frac{1}{\sqrt{2\pi}} Ss (\alpha_{-s}\sigma_{y_{x=-s}} - \alpha_{+s}\sigma_{y_{x=+s}}),$$

for all values of  $s$ ; while the sum product of the other half is the same expression with signs reversed.

Hence the correlations are equal and opposite.

Consequently a fraction of the whole surface would exhibit correlation absent from a fair sample of the whole surface. Evidently the comparison of

a sample of young children with one of older children is not a simple case of truncation such as here contemplated, but it seems to me possible that the principle operates.

*Intelligence.*

The corrected coefficients for intelligence are as follows. They differ considerably from those already given.

*Child in Fifth Year (entering school).*

Table						Partial coefficient
XX.	Age of mother at birth of child and her own standard	...	...	...	...	$r = -060 \pm .027$ $-079 \pm .027$
„	XXII.	Age of mother at birth and class of child	...	...	...	$r = -029 \pm .024$ $-024 \pm .028$
„	XXIV.	Standard of mother and class of child	...	...	...	$r = -074 \pm .024$ $-076 \pm .027$
„	XXVI.	Age of mother on leaving school and her own standard	...	...	...	$r = .435 \pm .022$ $.438 \pm .022$
„	XXVIII.	Age of mother on leaving school and class of child	...	...	...	$r = -010 \pm .028$ $-.023 \pm .028$
„	XXX.	Age of mother on leaving school and age at birth	...	...	...	$r = -.031 \pm .028$ $-.063 \pm .027$

*Child in Thirteenth Year.*

Table						Partial coefficient
XXI.	Age of mother at birth of child and her own standard	...	...	...	...	$r = -.1119 \pm .038$ $-.139 \pm .042$
„	XXIII.	Age of mother at birth and standard of child	...	...	...	$r = .110 \pm .041$ $.155 \pm .042$
„	XXV.	Standard of mother and standard of child	...	...	...	$r = .349 \pm .037$ $.389 \pm .036$
„	XXVII.	Age of mother on leaving school and her own standard	...	...	...	$r = .415 \pm .035$ $.430 \pm .034$
„	XXIX.	Age of mother on leaving school and standard of child	...	...	...	$r = .017 \pm .047$ $-.146 \pm .047$
„	XXXI.	Age of mother on leaving school and age at birth	...	...	...	$r = -.082 \pm .042$ $-.016 \pm .043$

The main points are, firstly, that the correlation between the standard of the mother on leaving school at a constant age with the class of the child in its 5th year is significant but very small, and the correlation between the standard of mother at constant age with standard of child at 13th year is very much larger, but not as large as might be expected. This may be due to errors of record or to the fact that the mental characters upon which scholastic intelligence depends, hardly exist at the 5th year and are not even fully developed at the 14th year.

If this explanation is adopted as correct, then intelligence falls into the same category as eye colour, that is to say, at the 5th year the scholastic intelligence of a child corresponds to the eye colour of a new born babe, and hence there is no significant correlation between age of mother at birth and class of child. At the 14th year we are dealing with a period during which intelligence is only half developed, that is to say, our record corresponds to eye colour during the first year and hence a significant correlation is found. Had our record been one dealing with a period of life when intelligence is as fully developed as it ever will be, then in all probability the correlation

would become insignificant. This assumes that intelligence follows the same lines as the other characters investigated, viz., that the chief effect of age of the uniting germ cells is to produce an increased variability in those upon which the time influence is the greater.

Still the difference between the value of the association between age of mother at birth and her own standard for the 5th year being  $+ \cdot 08$  and for the 13th  $- \cdot 14$ , definitely suggests that the two series differ in other ways, beyond the fact that the families concerned contain a child in the 5th-13th years. As was previously stated the data are not above suspicion of bias.

# REPORT OF BACTERIOLOGICAL INVESTIGATION OF TETANUS CARRIED OUT ON BEHALF OF THE WAR OFFICE COMMITTEE FOR THE STUDY OF TETANUS.

By W. J. TULLOCH, M.D., R.A.M.C., Bt. Major.

Lecturer in Bacteriology, University of St Andrews, Member of the War Office Committee  
for the Study of Tetanus.

(With 17 Diagrams.)

(From the Laboratories of the R.A.M. College and the Lister Institute  
of Preventive Medicine.)

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*To Major-General Sir DAVID BRUCE, K.C.B., F.R.S.,*  
Chairman of the War Office Committee for the Study of Tetanus.

SIR,

I have the honour to present the following Report dealing with the bacteriological research which has been prosecuted on behalf of the Tetanus Committee, during the period October 1917–October 1918.

Most of the work herein reported has been carried out jointly by Miss D. M. Cayley and myself.

Miss Cayley is solely responsible for Section VI (pp. 172–195) of the Report, in which is discussed the influence which various dressings and surgical procedures exert upon the anaërobic flora of wounds. The work was arduous and particularly trying, in that the information gained was by no means commensurate with the effort entailed in obtaining it. Miss Cayley is therefore to be congratulated upon her assiduous attention to this task.

The Report is divided into the following Sections:

I. An inquiry into the occurrence of the various types of *B. tetani* in the wounds of men suffering from tetanus.

II. An inquiry into the occurrence of the various types of *B. tetani* in wounds of men showing no evidence of tetanus.

III. A discussion of the facts set forth in Sections I and II.

IV. Experiments carried out *in vitro* to determine whether immune sera, prepared by inoculation of *whole culture* into animals, contained antibodies, other than agglutinins, specific to the "Types."

V. Experiments conducted *in vivo* to examine the problem of *infection* with *B. tetani*, as contrasted with *intoxication* due to absorption of the products of that organism.

The points dealt with in Section V are:

(a) The importance of a suitable *nidus* for the development of infection.

(b) Relation which the degree and nature of the tissue destruction or debilitation necessary for the inception of infection bears to the development of tetanus, and to prophylaxis by antitoxin.

(c) The part played by concomitant infection with micro-organisms other than *B. tetani* in activating or depressing the infective process and the intoxication in tetanus.

(d) The immunity conferred by the use of *mono-typical antitoxic* and *anti-bacterial* sera.

VI. The influence which various surgical procedures exert upon infection of wounds due to anaërobic bacteria.

VII. Attempts (a) to diagnose tetanus, and (b) to determine the "Type" of the infection in cases of the disease by means of an agglutination reaction, using the blood of patients suffering from tetanus for agglutinating stock emulsions of *B. tetani*.

VIII. An inquiry into the question of whether the serological Types are evolved as a result of the residence of the bacillus in the tissues of an individual belonging to one or other of the haemagglutinating groups of men.

IX. A discussion of two cases of abdominal tetanus.

X. The results of examination of faeces for the presence of *B. tetani* in the intestinal contents of

(a) Civilians,

(b) Men returned from active service.

Throughout each phase of the investigation one object only has been kept in view—the possible application of laboratory findings to the improvement of prophylaxis and therapeutics of tetanus.

Until *mono-typical antitoxic* and *anti-bacterial* sera are available in much larger quantities than is possible when ordinary laboratory animals are used for serum preparation, many of the questions raised by the work described in this Report must remain unsettled.

The investigations discussed in this Report are therefore admittedly incomplete, but the work has, I think, reached a phase in which it may, with advantage, be submitted for discussion.

I have the honour to be, Sir,

Your obedient Servant,

(Signed) WILLIAM J. TULLOCH, M.D., Bt. Major, R.A.M.C.

Lecturer in Bacteriology, The University of St Andrews.

#### INTRODUCTION.

IN a paper published in the *Journal of the R.A.M.C.*, December 1917, it was demonstrated, that *B. tetani* was susceptible of classification by serological methods into at least three "Types." Since the publication of that paper, a fourth Type has been encountered; but, so far, it has been demonstrated only in five instances—four being from cases of declared tetanus which were not, however, fatal.

The serological classification of *B. tetani* at once suggested the question: "What relationship, if any, do the various Types of the bacillus bear to the causation and pathology of the disease?"

The necessity for an inquiry was insistent, as both the prophylaxis and therapeutics of tetanus might have to be modified in view of the findings obtained.

The investigation was prosecuted as follows:

(a) As many cases of tetanus as possible were examined by bacteriological methods, in order to determine the serological Type of the bacillus responsible for the causation of the disease in each instance.

(b) In order to control these findings, the frequency with which the various serological Types of *B. tetani* could be obtained from wounds of men not suffering from tetanus, was made the subject of inquiry.

(c) Laboratory experiments, both *in vitro* and *in vivo*, were carried out, in order to determine whether crossed immunity to intoxication or to infection with the various Types, or their products, indicated that the typing of the bacilli was or was not of importance in relation to the pathogenesis of the disease and its serum prophylaxis. The question of the degree of passive immunity to *infection* conferred by the injection of antitoxin, naturally called for examination in this connection.

(d) The influence which various surgical procedures might have in preventing or lessening mass infection with anaërobes was also investigated. This Section of the work is of special interest and importance, as the most important element in the prevention of anaërobe infections is the surgical procedure employed in the treatment of wounds.

(e) The question was investigated as to whether diagnosis of the disease in its prodromal stage might not be assisted by an agglutination

technique, in which stock emulsions of the Type bacilli were exposed to patients' sera.

(f) The relationship which concomitant infection with certain of the commoner anaërobes—other than *B. tetani*—might bear to the causation of tetanus, was also made the subject of inquiry.

## SECTION I.

### TYPES OF *B. TETANI* OBTAINED FROM WOUNDS OF MEN SUFFERING FROM TETANUS.

This Section of the investigation was undertaken with a view to determining what influence, if any, universally applied serum prophylaxis exerted upon the incidence, course, and issue of the disease, in respect of the serological Types of the bacillus responsible for its causation.

From examination of cultures, seven in number, obtained from various Serum Institutes, it was found that all seven specimens conformed serologically to the "U.S.A. standard culture." As these seven cultures comprised those commonly in use in the English Serum Institutes for the preparation of tetanus antitoxin, it seemed not improbable, that serum. corresponding to one serological type of the bacillus only, was being employed for prophylaxis, so far as the bulk of our own troops was concerned. This might result in the elimination of the disease produced by one serological Type of the bacillus, but might not, to the same extent, reduce the incidence of the disease, when the causal organism was *heterologous* to the prophylactic serum.

This appeared to be strikingly borne out by the examination of the first 25 strains of *B. tetani* obtained from cases of the disease, as typed by the agglutination method. Only one belonged to the same serological group as the standard U.S.A. culture—hereafter referred to as "Type I." At the same time, of the four cultures of toxic tetanus bacilli that had been then isolated from wounds of men not suffering from the disease, three conformed to that Type on serological investigation.

It will be seen from the further consideration of this Section of the Report, that although the hypothesis tentatively advanced above is possibly correct, the demarcation between cases due to Type I bacilli and cases due to the other Types among inoculated men, is not so clear cut as the earlier inquiries might have led one to believe.

#### (a) *Fallacies of investigation.*

In making this investigation, the number of fallacies which may be introduced is such, that any figures obtained and quoted can only have a relative value. Apart from the technical difficulties which have to be overcome in attempting to obtain the organism in a sufficient state of purity to permit of the application of serological tests, other quite uncontrolled sources of error



affect the problem. Great difficulty is, therefore, experienced in interpreting the results obtained.

The present series of cases, 100 in number, represents the successful results obtained in the examination of about 200 specimens. It may seem that a 50 per cent. rate of successful investigations is low, but it must be borne in mind, that the material was not collected by the bacteriologist, and that it was forwarded to the laboratory often from long distances. The investigator was therefore not in a position to obtain optimum results.

Among the cases in which I failed to obtain suitable growths for agglutination, a small number of swabs grew no organisms at all; a larger number grew only aërobes, and in a still larger number there were no organisms resembling *B. tetani*, either in meat-water cultures or in cultures made in media of a more complex composition. A considerable number of the cultures which did develop bacilli bearing a spherical terminal spore, failed to agglutinate in presence of any of the four Type sera. The majority of these agglutinated in presence of a serum prepared by inoculating an animal with a culture of a non-toxic round end-sporing bacillus, whose precise position and relationship have not yet been determined.

In point of fact, it is remarkable, in view of the technical difficulties that are encountered, that not so few, but so many as 50 per cent. were successfully investigated. This purely technical difficulty then, introduces an experimental error, the assessment of which is by no means easy. This error can only be reduced by examination of a further series of cases. In addition to the difficulty of isolation, the occurrence in the cultures of a peculiar inagglutinable phase renders statistical inquiry, based on agglutination, a laborious and not completely satisfactory procedure.

I wish here to emphasise a point that might otherwise be misunderstood, viz., that I do not consider it probable that all organisms capable of elaborating spasm-producing toxins, will necessarily be agglutinated by one or other of the four Type sera; I do think it probable, however, that the majority of such organisms can be so agglutinated.

In any series of cases of tetanus, the incidence, course, and termination of the disease are all modified by a number of factors. These factors, which in each individual case will modify the disease, are:

(i) The nature of the wound, the extent of the solution of continuity, the involvement of muscle or other structures, the degree of devitalisation of tissue due to direct trauma, the degree of interference with the nutrition of the part due to concomitant trauma to vessels, etc.; all these must play a part, and an important part, in the pathogenesis and final issue of any case. Short of visiting and personally examining every case, the part played by each of these factors in the causation of the disease cannot be even remotely estimated.

(ii) The position of the wound and its anatomical relationship may have some bearing on the incidence and result of infection with *B. tetani*.

(iii) The nature and extent of concomitant infection with other organisms is an extremely important, and a quite uncontrollable (uncontrollable from the standpoint of the investigator) factor in determining the onset of tetanus. This question is dealt with to some extent in Section V of the Report.

(iv) As a natural corollary of (a) and (c) *supra*, the nature of the first surgical interference carried out immediately after the reception of the wound, and, to a less extent, the procedure thereafter followed, will influence profoundly the subsequent history of a case in respect of the incidence of all specific infection—in the present instance infection due to gas gangrene bacilli and *B. tetani*.

(v) The conditions under which the wound was received, notably, the length of time the man lay out before he was picked up, will also have a bearing upon the subsequent development of tetanus and other infections.

(vi) Other conditions, which cannot be dismissed without consideration in this connection, are meteorological, geographical, geological, seasonal and agricultural conditions.

With so many potential sources of error intrinsic to the cases and inseparable from the technique that one is forced to adopt, the present series of 100 cases cannot be expected to give unequivocal information on the point of issue. None the less, the series is of interest and suggests certain not unimportant considerations.

(b) *Tabulation of cases due to various types.*

Table I shows the number of cases, the incubation period, and the final result in those instances, in which Type I bacilli were recovered from wound exudates of cases of declared tetanus.

Table II shows the same with regard to Type II bacilli.

Table III shows the same with regard to Type III bacilli.

Table IV shows the same with regard to Type IV bacilli.

Table I. *Cases of Tetanus due to Type I.*

Recovery or death	Prophy- lactic	Thera- peutic	Local	General	Where wounded	Onset
1. Recovered	+	+	...	+	St Quentin ...	7 days
2. Recovered	+	+	...	+	Delville Wood ...	7 "
3. <i>Died</i>	+	+	...	+	Vieux Berquin ...	7 "
4. <i>Died</i>	-	+	...	+	Peronne ...	8 "
5. Recovered	+	+	...	+	... ..	9 "
6. <i>Died</i>	-	+	...	+	Hull Docks ...	9 "
7. Recovered	+	+	...	+	France ...	9 "
8. Recovered	+	+	...	+	Delville Wood ...	10 "
9. <i>Died</i>	+	+	...	+	Near Maillet ...	10 "
10. Recovered	+	+	+	...	Posièrès ...	11 "
11. <i>Died</i>	+	+	...	+	Somme ...	11 "
12. Recovered	+	+	+	...	Bon Hamel ...	11 "
13. <i>Died</i>	+	-	+	...	Amiens ...	12 "
14. Recovered	+	+	+	...	Thiepval ...	13 "
15. Recovered	+	+	...	+	Somme ...	13 "

Table I. *Cases of Tetanus due to Type I—(contd.)*

Recovery or death	Prophylactic	Therapeutic	Local	General	Where wounded	Onset
16. Recovered	+	+	...	+	Ypres ...	13 days
17. Recovered	+	+	...	+	Mametz Wood ...	14 "
18. Recovered	+	+	+	...	Corbie ...	16 "
19. <i>Died</i>	+	+	...	+	Thiepval ...	16 "
20. <i>Died</i>	+	+	...	+	Cambrai ...	17 "
21. Recovered	+	+	+	...	La Boisselle ...	18 "
22. Recovered	+	+	...	+	Le Transloy ...	19 "
23. Recovered	+	+	...	+	Bapaume ...	19 "
24. Recovered	+	+	...	+	Bullecourt ...	19 "
25. Recovered	+	+	...	+	Somme ...	20 "
26. Recovered	+	+	...	+	Roye ...	21 "
27. Recovered	+	+	...	+	Bazantin le petit ...	21 "
28. Recovered	+	+	...	+	Gouzicourt ...	22 "
29. Recovered	+	+	...	+	Hardicourt ...	29 "
30. <i>Died</i>	-	+	...	+	Armentières ...	33 "
31. Recovered	+	+	...	+	Albert ...	33 "
32. Recovered	+	+	...	+	Ypres ...	46 "
33. Recovered	+	+	...	+	Neuve Chapelle ...	54 "
34. Recovered	+	+	...	+	Ypres ...	54 "
35. Recovered	+	+	...	+	... ..	56 "
36. Recovered	+	+	...	+	Somme ...	58 "
37. Recovered	+	+	...	+	Fricourt ...	75 "
38. Recovered	+	+	...	+	Ypres ...	94 "
39. Recovered	+	+	...	+	Somme ...	101 "
40. Recovered	+	+	...	+	Poelcapelle ...	110 "
41. Recovered	+	+	...	+	Polygon Wood ...	114 "

Table II. *Cases of Tetanus due to Type II.*

Recovery or death	Prophylactic	Therapeutic	Local	General	Where wounded	Onset
1. Recovered	-	+	...	+	Newton Hungerford	7 days
2. Recovered	+	+	+	...	Hollebeke ...	8 "
3. Recovered	+	+	...	+	... ..	8 "
4. <i>Died</i>	+	+	...	+	Vimy Ridge ...	9 "
5. Recovered	+	+	+	...	Ypres ...	10 "
6. <i>Died</i>	+	+	...	+	Hirnville ...	10 "
7. <i>Died</i>	+	+	...	+	... ..	10 "
8. Recovered	+	+	+	...	Ypres ...	12 "
9. Recovered	+	+	...	+	Poelcapelle ...	12 "
10. <i>Died</i>	-	+	...	+	France ...	14 "
11. Recovered	+	+	+	...	Zonnebeke ...	15 "
12. Recovered	+	+	...	+	Guinchy ...	16 "
13. <i>Died</i>	+	+	...	+	... ..	18 "
14. <i>Died</i>	+	+	...	+	France ...	19 "
15. <i>Died</i>	-	+	...	+	Abergelo (Wales) ...	19 "
16. <i>Died</i>	+	+	+	...	Ypres ...	21 "
17. Recovered	- ?	+	+	...	Posières ...	28 "
18. Recovered	+	+	+	...	Ypres ...	76 "
19. Recovered	+	+	...	+	Langemarke ...	77 "
20. Recovered	+	+	...	+	Vimy Ridge ...	120 "

21. Details not available.  
22.

Table III. *Cases of Tetanus due to Type III.*

Recovery or death	Prophylactic	Therapeutic	Local	General	Where wounded	Onset
1. <i>Died</i>	+	†	...	+	Vimy Ridge ...	3 days
2. <i>Died</i>	+	+	...	+	France ...	5 "
3. <i>Died</i>	+	+	...	†	Cambrai ...	6 "
4. Recovered	†	+	...	+	Villers Bretonneux	6 "
5. <i>Died</i>	+	+	...	+	Thiepval ...	7 "
6. <i>Died</i>	-	†	...	+	Cambrai ...	8 "
7. Recovered	+	+	...	+	Albert ...	8 "
8. <i>Died</i>	-	+	...	†	Yorkshire ...	8 "
9. Recovered	+	+	...	+	Bullecourt ...	9 "
10. <i>Died</i>	†	†	...	+	... ..	10 "
11. Recovered	+	†	+	...	Amiens ...	10 "
12. Recovered	†	†	+	...	Ypres ...	11 "
13. <i>Died</i>	†	+	...	+	Borsinghe ...	12 "
14. Recovered	+	+	...	+	... ..	13 "
15. <i>Died</i> *	+	+	...	+	Villers Bretonneux	14 "
16. Recovered	+	+	...	†	Bullecourt ...	14 "
17. <i>Died</i>	+	+	...	†	Trones Wood ...	14 "
18. Recovered	+	+	...	†	Trones Wood ...	15 "
19. <i>Died</i>	+	+	...	†	St Quentin ...	16 "
20. Recovered	+	+	...	†	Paschendaele ...	17 "
21. Recovered	+	+	+	...	Paschendaele ...	18 "
22. Recovered	+	†	...	+	Ypres ...	18 "
23. <i>Died</i>	+	†	...	†	... ..	19 "
24. Recovered	+	+	...	+	Amiens ...	20 "
25. <i>Died</i>	+	+	...	+	Somme ...	30 "
26. Recovered	†	+	...	+	Gonnelieu ...	30 "
27. <i>Died</i>	†	+	...	+	Guinchy ...	43 "
28. Recovered	+	+	...	+	Blighty Wood ...	54 "
29. Recovered	+	+	...	+	Albert ...	57 "
30. Recovered	+	+	...	+	Guyencourt ...	65 "
31. Recovered	+	+	...	+	France ...	81 "
32. Recovered	+	+	...	+	Cambrai ...	101 "
33. <i>Died</i>	+	+	...	+	... ..	149 "

\* Prophylactic inoculation made late.

Table IV. *Cases of Tetanus due to Type IV.*

Recovery or death	Prophylactic	Therapeutic	Local	General	Where wounded	Onset
1. Recovered	-	+	+	...	Posières ...	4 days
2. Recovered	+	+	...	+	Villers Pouchée ...	17 "
3. Recovered	+	+	...	+	Bapaume ...	48 "
4. Recovered	+	+	...	+	Ypres ...	49 "

The findings described in Tables I, II, III, and IV, are graphically summarised in the following Diagram I (p. 112).

Commenting on these results, I wish to call particular attention to a point of great importance, viz., that the prophylactic administration of A.T.S., although failing to prevent absolutely the occurrence of tetanus, tends very markedly to reduce the death-rate from the disease. This is indicated by the fact that death occurred in six out of seven cases of generalised tetanus in

the present series, which did *not* receive a prophylactic dose of A.T.S. Whereas of 75 cases, which were definitely known to have received a prophylactic dose of serum, only 25 terminated fatally.

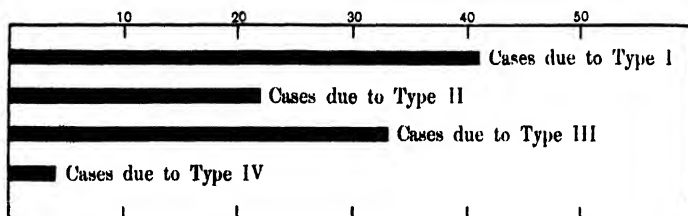


DIAGRAM I. Types of *B. tetani* obtained from 100 cases of tetanus in which the organism was cultured in a sufficient state of purity to permit of serological examination.

If now, we limit ourselves to those cases which received a prophylactic injection of serum the following figures are obtained.

(a) Type I appeared to be the organism responsible for the disease in 38 cases; six deaths occurred in this series, of which four were definitely attributable to tetanus, one was certainly not due to that disease, and in the remaining case the cause of death was doubtful. This gives a death-rate of 13.1 per cent. for cases due to Type I.

(b) Type II was found in 18 cases, six of which terminated fatally. In five instances death was definitely attributable to tetanus—a death-rate for Type II of 27.7 per cent.

(c) Of Type III there were 31 cases with 12 deaths, all of which were attributable to tetanus. In one case the prophylactic dose of A.T.S. was known to have been administered late. Therefore, we may regard the death-rate as 11 out of 31 (= 35.5 per cent.).

(d) Of Type IV infection there were only four cases none of which were fatal.

These facts are presented graphically in Diagram II.

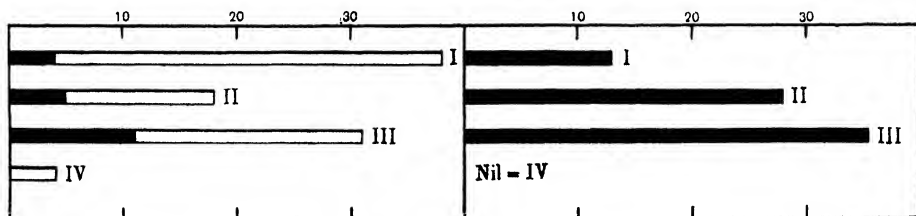


DIAGRAM II. Number of each type of *B. tetani* recovered from declared cases of the disease occurring in inoculated men.

The actual number of deaths due to each type

= ■■■■

Deaths from each type of infection expressed as a percentage of the number of cases in which each type was "isolated" from declared cases of tetanus in inoculated men.

These findings call for the following comments.

(c) *Discussion of findings.*

(a) Owing to the total number of cases examined (100) being small, it is difficult to draw definite conclusions from the results obtained. I think, however, that the findings are suggestive, particularly in view of the fact that, both in the case of Type II and in that of Type III infections, the death-rate is considerably higher than when Type I is the organism responsible for the disease.

(b) Although Type I bacilli appear to be responsible for a larger number of cases of tetanus than are Types II or III, the death-rate among inoculated men from Type I is lower than when Type II or Type III is the infecting organism.

This might be due simply to there being a relatively larger number of strains of Types II and III virulent to man than there are of Type I; on the contrary, it may mean that the serum used for prophylaxis affords more adequate protection against infection with Type I bacilli than against infection with Types II and III. Further Sections of the present Report deal with attempts which have been made to determine which of these hypotheses is correct.

The figures, so far obtained, are, however, susceptible of inquiry from another point of view. If the low death-rate and (as will be seen from Section III of the present Report) the relatively low rate of incidence of cases due to Type I infection, are attributable to special qualities of the serum used for prophylaxis, then, the number of cases occurring during the first two weeks after wounding, and the proportion of those which terminated fatally, should be relatively greater in infections due to Types II and III than in those due to Type I.

On examining Tables I, II and III, it will be noted that:

1. Of 38 cases of Type I infection in inoculated men the onset of the disease occurred within 14 days in 15 instances, and death was attributable to tetanus in three out of 15 instances.
2. Of 17 cases in inoculated men in which the causal organism was a Type II bacillus, eight occurred within the 14-day period and of these three proved fatal.
3. Of Type III cases there are 31 in inoculated men; in 15 instances the onset occurred within the 14-day period and eight of them proved fatal. Death was attributable to tetanus in all eight cases.

These results may, for purposes of comparison, be graphically expressed thus.

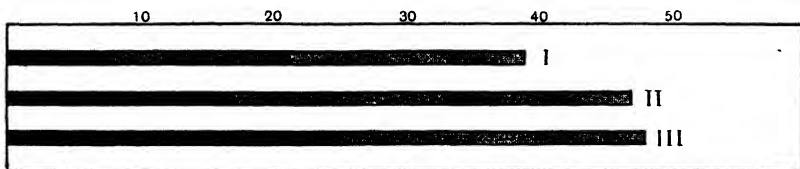


DIAGRAM III. Cases of tetanus in inoculated men due to Types I, II, and III in which onset occurred within 2 weeks. Rates expressed as percentages.

■ = Incidence. ■ = Death rate.

Regarded superficially, these figures do not appear to carry conviction, as the numbers dealt with are too small.

A more careful consideration of them, however, indicates that they are extremely suggestive, and a fairly definite interpretation of these figures is made possible by the results obtained in Sections III and V of this Report. Section III shows that Type I bacilli are found much more frequently than are Types II or III in wounds of (inoculated) men who show no evidence of tetanus; while Section V demonstrates that, in passively immunised animals, the development of tetanus infection depends to a preponderating extent upon the degree of tissue destruction occurring at the time of injury. It is only natural then, that Type I cases can and will occur within the 14-day period, and that a certain number of these will prove fatal; for, if the degree of tissue destruction exceeds certain limits, no amount of antitoxin—in practical dosage at least—will prevent the occurrence of the disease.

That the findings obtained in this Section of the work represent a fair average of what would probably be found in examining a larger series of cases is suggested by the fact, that, of about 100 cases of tetanus occurring in inoculated men, from whose wounds I failed to obtain *B. tetani* in a sufficient state of purity to permit of its serological examination, the death-rate was 21 per cent., whereas in the series under discussion, the death-rate is much the same—25 per cent.

#### GENERAL CONCLUSIONS FROM SECTION I (pp. 107–114).

An examination of 100 cultures of *B. tetani* obtained from wounds of men suffering from tetanus shows:

1. That the value of serum prophylaxis is very great indeed. Practically all the cases of the disease which occur in men who have not received serum prophylaxis terminate fatally.
2. There are indications both from
  - (a) The higher death-rate, and
  - (b) The earlier onset of the disease among men who did receive serum prophylaxis, that, either, Types II and III are more virulent for man than is Type I, or, the protection afforded by the serum at present in use, is more adequate against infection due to Type I bacilli than to Types II or III.

#### SECTION II.

##### TYPES OF TETANUS BACILLI RECOVERED FROM MEN SHOWING NO EVIDENCE OF TETANUS.

An investigation into the distribution of the various serological Types of *B. tetani* in wounds of men who show no clinical evidence of tetanus is an essential corollary to the previous inquiry. If such an investigation be not made, wrong interpretations might be put upon the findings of Section I,

and a false perspective might be obtained of the actual incidence of the various Types of the bacillus in wounds. During the period in which the series of cases discussed in Section I was being submitted to bacteriological examination, a number of swabs were forwarded to the laboratory from wounds in which organisms resembling *B. tetani* had been demonstrated by other investigators, although the patients showed no evidence of tetanus.

These were submitted to complete bacteriological examination along with cultures containing bacilli resembling *B. tetani* which Miss Cayley encountered in making an investigation into the anaërobic flora of a series of 100 wounds.

In all, 25 such strains were obtained from wounds. These 25 strains were each carefully examined and only qualified as members of one or other of the serological Types of *B. tetani* when they

- (1) agglutinated in presence of one or other of the agglutinating sera,
- (2) produced toxin when grown *in vitro*, or,
- (3) caused tetanus when injected together with a tissue-debilitant.

It may seem peculiar that a differentiation is made between

- (a) power to produce toxin when grown *in vitro*, and
- (b) power to produce the disease when washed spores of the culture are injected together with a tissue-debilitant.

But, however, such differentiation of these two factors must be made, in view of the findings which are discussed *in extenso* in Section V of the present Report.

Of these 25 strains which were proved both by agglutination and animal experiment

19 were Type I bacilli

3    "    "    II    "

2    "    "    III    "

1 was    "    IV    "

The results obtained in Section II are graphically shown in the following diagram—Diagram IV.

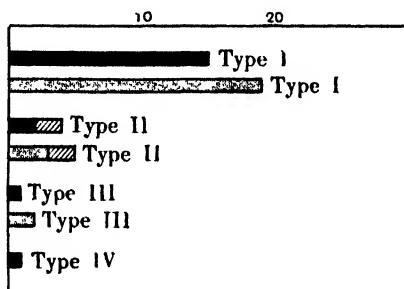


DIAGRAM IV. Actual numbers of *B. tetani*, proved by animal tests and obtained from wounds of men showing no evidence of tetanus.

■ = From series of 100 wounds.  
 ■ = From all sources.  
 ■ = Result equivocal.



The details of the examination of each of these strains are given in the following Table.

Table V.

*A. Strains of B. tetani obtained from the examination of 100 wounds of men not suffering from Tetanus.*

No.	Animal experiment	Agglutination	Since wounding	Where wounded
1.	Toxin expt. positive	Type I	7 days	Ypres
2.	" "	" I	7 "	"
3.	" "	" I	20 "	Albert
4.	Toxin expt. negative Infection expt. positive	" I	24 "	"
5.	Toxin expt. positive	" I	25 "	Villers Bretonneux
6.	" "	" I	30 "	Mericourt
7.	Toxin expt. negative Infection expt. positive	" I	32 "	Cambrai
8.	Toxin expt. negative Infection expt. positive	" I	35 "	"
9.	Toxin expt. negative Infection expt. positive	" I	39 "	"
10.	Toxin expt. positive	" I	64 "	Baersee
11.	" "	" I	112 "	Ypres
12.	" "	" I	130 "	Cambrai
13.	See Footnote (i)	" I	733 "	Hohenzollern Redoubt
14.	Toxin expt. positive	" I	860 "	Poelcapelle
15.	" "	" I	882 "	Cambrai
16.	" "	" II	15 "	Bapaume
17.	See Footnote (ii)	" II	100 "	Cambrai
18.	Toxin expt. positive	" II	138 "	Passchendaele
19.	See Footnote (ii)	" II	54 "	Monchy
20.	Toxin expt. positive	" III	16 "	Orvillers
21.	" "	" IV	305 "	Hermies

*B. Strains of B. tetani obtained from wounds of men not suffering from Tetanus obtained from various other sources.*

1.	Toxin expt. positive	Type I	49 days	Cambrai
2.	" "	" I	56 "	"
3.	" "	" I	56 "	"
4.	" "	" I	No details obtainable	
5.	" "	" II	8 days	No further details
6.	" "	" III	No details obtainable	

*Footnote (i).* With reference to No. 13, toxin experiments were negative, and when an attempt was made to carry out the infection experiment, the animals died of gas gangrene.

*Footnote (ii).* Culture 17 which agglutinated in presence of Type II serum was lost before the animal experiments were completed; culture 19 is still under investigation.

If we limit ourselves to the consideration of those cultures obtained by Miss Cayley in the examination of 100 wounds in Table I A, it is found that

Type I bacilli were found in 15 cases

„ II	„	„	2	„
„ III	„	„	1	case
„ IV	„	„	1	„

In addition to the two cultures of Type II recorded above there were the two other cases, namely cultures 17 and 19, which could not be fully investigated.

The results dealt with in this Section will be more fully discussed in Section III.

Commenting on these results it is seen:

1. That Type I bacilli appear to be much more frequently found in wounds than are bacilli of the other serological Types.

2. That, in 100 wounds of men who showed no evidence of tetanus, *B. tetani* could be recovered in certainly 19 and probably in 21 instances.

### SECTION III.

THE RESULTS OBTAINED IN SECTION I AND SECTION II ARE CONTRASTED.

NOTE ON THE GEOGRAPHICAL DISTRIBUTION OF THE VARIOUS SEROLOGICAL TYPES OF *B. TETANI* ON THE WESTERN FRONT.

If the findings of Section I, which deals with the investigation of swabs from wounds of men suffering from tetanus, be contrasted with the findings of Section II, which deals with the demonstration of tetanus bacilli in the wounds of men showing no evidence of tetanus, the following results are obtained.

The total number of tetanus bacilli isolated from cases of the disease = 100.

The total number of tetanus bacilli isolated from wounds of men showing no evidence of tetanus = 25.

Type I from cases	=41	41 %
„ I „ indifferent wounds	=19	76
„ II „ cases	=22	22
„ II „ indifferent wounds	= 3	12
„ III „ cases	=33	33
„ III „ indifferent wounds	= 2	8
„ IV „ cases	= 4	4
„ IV „ indifferent wounds	= 1	4

This is graphically shown in Diagram V.

*Note.* In Diagram V, the open column referring to Type II indicates the percentage of proved Type II bacilli obtained from wounds of men not suffering from tetanus.

The hatched column indicates the Type II cultures from the same source in which the investigation is not completed.

These findings also suggest that the protection afforded by the serum at present in use is more adequate in respect of Type I infection than in respect of infections due to Types II and III. The number of Type IV cases is as yet too small to permit of any deductions being made from the results obtained.

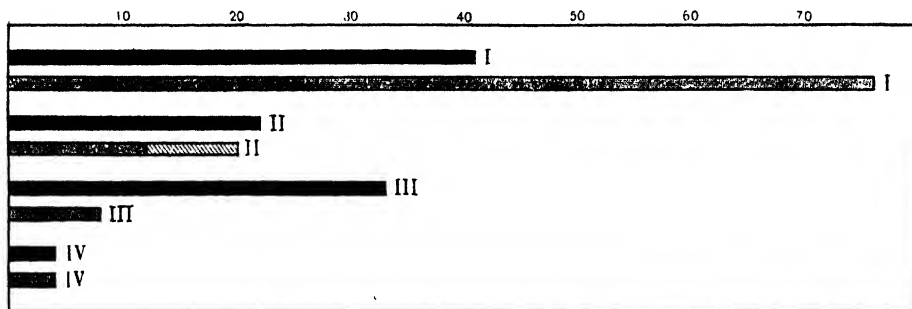


DIAGRAM V. Types of *B. tetani* "isolated" from cases of the disease compared with those "isolated" from "indifferent" wounds each expressed as percentage of the total numbers "isolated" from each source.

■ = From cases.

■ = From "indifferent" wounds.

▨ = Type II bacilli from "indifferent" wounds the examination of which was not completed or the result obtained was equivocal.

The figures so far obtained may be dealt with from another standpoint. We know that 20 per cent. of wounds may contain tetanus bacilli, it is also known that the incidence of tetanus among the wounded in England is roughly 1 per 1000. From these figures it appears that in 1000 wounds there are 160 which are infected with Type I bacilli, and therefore, only 1 in 160 infections with that organism gives rise to declared tetanus.

On the same basis 1 in 20 at the lowest, or 1 in 40 at the highest estimate, gives rise to the disease in the case of Type II infections. Of Type III infections there appear to be only 10 per 1000, and therefore, every tenth man infected with Type III bacilli may fall a victim to the disease.

It is questionable if this reasoning is strictly justifiable, for the assumption is made that three cases instead of one have occurred per 1000 wounded men. As the results, however, are only of comparative and not of absolute significance, the same error is introduced in each instance.

The suggestion which arises from the consideration of the facts from this point of view may be diagrammatically shown thus (Diagram VI, p. 119).

The following conclusions appear, therefore, to be permissible from the comparison of the results of the investigations dealt with in Sections I and II.

1. That Type I bacilli are of more frequent occurrence in nature than are Types II and III.

2. That the serum at present in use for prophylaxis affords more adequate protection against Type I infection than against infection with the other Types.

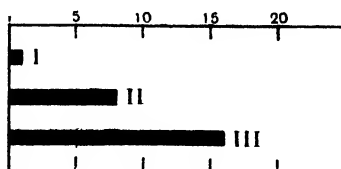


DIAGRAM VI. The columns indicate that of 160 men infected with

Type I bacilli,	1	would take the disease
Type II	8	„ „ „
Type III	16	„ „ „

These deductions are made with reserve, as so many factors enter into the causation of the natural disease in man, and so many more appear to affect the toxogenicity of *B. tetani*. Short of unequivocal experimental evidence being obtained, that more adequate protection is afforded by a serum *homologous* to the infecting bacillary Type, than by a serum *heterologous* thereto, one cannot arrive at a definite conclusion concerning the point at issue.

#### GEOGRAPHICAL DISTRIBUTION OF VARIOUS TYPES OF *B. TETANI* ON THE WESTERN FRONT.

As a matter of scientific interest, if not of practical importance, a record was kept of the districts in France where each man received his injury. In 97 instances this information was available. If the area occupied by British troops be divided roughly into two districts, (1) north of the La Bassée Canal, and (2) south of La Bassée Canal, it is found that:

(i) of 50 strains of Type I bacilli		13	came from the N Area	
		37	„	„ S „
(ii)	„ 15 „ „ II „	11	„	„ N „
		4	„	„ S „
(iii)	„ 27 „ „ III „	6	„	„ N „
		21	„	„ S „
(iv)	„ 5 „ „ IV „	1	„	„ N „
		4	„	„ S „

These figures suggest that the various serological Types may have different geographical distribution in view of the relatively large number of Type II bacilli which were obtained from Flanders. This is further borne out by the fact that during the period of the Flanders offensive Type II were frequently found, while with the alteration of the fighting to the Somme area, Types I and III were those commonly obtained.

It is only fair to note that among a small number of cultures obtained from men who received injuries in this country all types of *B. tetani* were represented.

## SECTION IV.

DEMONSTRATION OF ANTIBODIES, OTHER THAN AGGLUTININS, SPECIFIC TO THE SEROLOGICAL TYPES OF *B. TETANI*.

While the previous Sections of this Report suggest that the incidence of tetanus among inoculated men may to some extent depend upon the type of the infecting bacillus, and the serological relationship which it bears to the organism used for the preparation of prophylactic antitoxin, the findings by no means prove that there is such a relationship. In the foregoing Sections stress has been laid upon the difficulty of interpreting the results obtained by making a statistical inquiry into the problem, and these difficulties are again here emphasised.

It was necessary therefore to prosecute experimental investigation while the other phases of the work were proceeding.

A. *The first question which naturally arises is:* "Is the toxin of one serological Type of the bacillus more adequately neutralised by its own *specific* antitoxin than by antitoxin produced by inoculation of the product of a serologically *heterologous* bacillus?"

The findings of Leuchs (*Zeitschrift für Hygiene*, 1918, Bd 65, p. 55) in respect of the toxins of different strains of *B. botulinus* suggested that quantitative differences, at least, might exist between the soluble toxins of the various Types in relation to the neutralising value of an antitoxin corresponding to any one Type.

Experiments were therefore made with a view to examining this point. The results obtained showed definitely:

(a) That no *qualitative* difference existed between the toxins produced by the bacillus of the different Types.

One antitoxin neutralised the toxin of any Type or of all Types.

(b) That, if a *quantitative* difference exist, it is so slight, that when mice or rats are the animals employed for making these tests, no quantitative relationship of a specific nature can be demonstrated.

B. *The second question which therefore arises is:* "As the soluble toxins of all Types of *B. tetani* are the same, but, as the bacilli themselves are different one from another as *antigens*, is this *antigenic* difference of the bacilli of any import in their pathogenic property, and is it of special importance in the unnatural conditions arising from universally applied serum prophylaxis?"

It seemed not improbable, that the value of antitoxin for prophylactic purposes might depend upon more than one factor:

(1) We know that one factor is the capacity for neutralising the *spasm-producing* toxin of all tetanus bacilli.

(2) Hypothetically, another factor that might be considered is the capacity for preventing *infection*.

The former of these factors we have demonstrated to be non-specific in respect of the Types—at least in so far as the experimental animals employed are concerned. The second (hypothetical) factor, which has not hitherto been inquired into in a systematic way, may be specific. If this be the case, a *mono-typical* serum would protect most adequately against infection with a serologically *homologous* organism.

The inquiry therefore resolved itself into the investigation of the following problems:

(a) Can antibodies other than “agglutinins,” which are *specific* to the Types, and “anti-spasmins” which are *not specific* to the Types, be evoked by inoculation of cultures of the bacilli or of their products?

(b) If such specific antibodies can be demonstrated, and serum containing them be proved to be of prophylactic value, how should we proceed to prepare sera containing these antibodies?

*Note.* The term “proved to be of prophylactic value” means, proved to prevent *infection* which will cause death in the unprotected animal or in the animal inadequately protected by antitoxin.

(c) What relationship do the various demonstrable antibodies bear to one another?

(d) What is the mechanism of tetanus *infection* as opposed to tetanus (spasm-producing) *intoxication*?

To investigate these questions two separate and distinct methods of inquiry were followed:

(1) *In vitro* experiments were carried out with various sera and cultures.

(2) Infection experiments *in vivo*.

This Section of the Report (No. IV) deals only with the *in vitro* experiments.

#### (a) *Preparation of sera.*

Nine rabbits were immunised as follows:

I received a course of immunisation with the filtered toxin of a *two-day culture* of Type I bacilli.

The toxin was stored for six weeks in the ice-chest before use, and a *two-day toxin* was chosen so that the least possible autolysis of the bacillus had occurred, and yet an adequate toxin was obtainable.

II was immunised by inoculating intravenously *whole culture* of Type I bacilli grown for 24 hours.

III underwent a course of immunisation with *washed* Type I bacilli.

I'	corresponded to I but Type II toxin was used	
II'	II	II culture was used
III'	III	II washed bacilli used
I''	I	III toxin was used
II''	II	III culture was used
III''	III	III washed bacilli used.

The rationale of choosing the three methods of immunisation was that:

(a) The animals inoculated with toxin would produce antibodies to the filtrable antigens, and, as stored toxin was used, only antibodies to those antigens which were stable.

(b) The animals immunised with all the products of a young culture would produce antibodies to:

(i) any spasm-producing toxin that might have developed,

(ii) to the bacilli themselves,

(iii) to any soluble but unfiltrable or unstable antigens which the organism might develop during its phase of active growth.

It might be argued that it is improbable that a soluble but unfiltrable antigen would be elaborated during the growth of any organism; but if the filtration experiments dealing with the products of pathogenic anaërobes in general be scrutinised, one notes that difficulty has been experienced owing to the large amount of active product that is absorbed in filtration, and filtrates deficient in toxicity have been obtained.

Young cultures were designedly chosen for immunising animals II, II', II'', because it is known, that certain of the pathogenic anaërobic bacilli—notably *B. Welchii*—elaborate tissue-debilitating poisons during their period of active growth. These poisons are evanescent, and the toxicity of cultures markedly declines on continued incubation for several days at 37° C.

It seemed possible that *B. tetani* might give rise to similar aggressive products that have not so far been demonstrated, because they were not sought for.

(c) The animals inoculated with *washed* cultures should produce only antibodies to the bacillary substance.

These nine animals, after having been under immunisation for approximately two months, were bled by Durham's technique to the extent of about 10 c.c. each and a complete series of observations was made with the sera so obtained.

(b) *Preliminary investigation of sera.*

*Firstly, the antitoxic titre was roughly determined.*

Rabbit No. I—approximately 12 units per c.c.

„ II „ 2 „ „

„ III „ 1 unit „

*Note.* Animal No. III, owing to an accident, received a number of inoculations with bacilli that had been washed in saline only once, while No. III' and III'' were immunised with bacilli which had been washed several times.

No. I' approximately 5 units per c.c.

„ II' „ 2 „ „

„ III' „ less than 0.5 per c.c.

„ I'' „ 12 units per c.c.

„ II'' „ 2 „ „

„ III' „ less than 0.5 per c.c.

Secondly, the agglutinin titre of each was determined, and cross experiments with all three Types of bacilli were carried out.

This Section of the work is of considerable importance for two reasons:

(i) A perfectly just criticism might be made of a previous communication on the serological differentiation of tetanus bacilli *inter se* (*Journal of R.A.M.C.*, Dec. 1917), viz. that owing to the low titre of the serum then

Table VI.

Serum No. I = Type I Toxin							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Type I	-	-	-	-	-	-	-
„ II	-	-	-	-	-	-	-
„ III	-	-	-	-	-	-	-
Serum No. II = Type I 24-hour Whole Culture							
Type I	+	+	+	+	+	+	-
„ II	-	-	-	-	-	-	-
„ III	-	-	-	-	-	-	-
Serum No. III = Type I Washed Bacilli							
Type I	+	+	+	+	+	-	-
„ II	-	-	-	-	-	-	-
„ III	-	-	-	-	-	-	-
Serum No. I' = Type II Toxin							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Type I	-	-	-	-	-	-	-
„ II	-	-	-	-	-	-	-
„ III	-	-	-	-	-	-	-
Serum No. II' = Type II 24-hour Whole Culture							
Type I	-	-	-	-	-	-	-
„ II	+	+	+	+	+	+	-
„ III	-	-	-	-	-	-	-
Serum No. III' = Type II Washed Bacilli							
Type I	-	-	-	-	-	-	-
„ II	+	+	+	+	+	+	-
„ III	-	-	-	-	-	-	-
Serum No. I'' = Type III Toxin							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Type I	-	-	-	-	-	-	-
„ II	-	-	-	-	-	-	-
„ III	-	-	-	-	-	-	-
Serum No. II'' = Type III 24-hour Whole Culture							
Type I	-	-	-	-	-	-	-
„ II	-	-	-	-	-	-	-
„ III	+	+	+	+	+	-	-
Serum No. III'' = Type III Washed Bacilli							
Type I	-	-	-	-	-	-	-
„ II	-	-	-	-	-	-	-
„ III	+	+	+	+	+	+	-



employed—1/400—the specific agglutination results therein figured might give a false impression concerning the complete specificity of the Types of *B. tetani* from the standpoint of agglutination. The low titre of the serum might unduly enhance the specificity of the tests.

(ii) The cultures used for inoculating these animals had been isolated a year previously, and, since isolation, had been frequently sub-cultured—sometimes daily over long periods.

I call attention to this, because it had been suggested that serologically different races of the bacillus might be evolved as a result of residence in human tissue or in close proximity thereto.

Table VI illustrates the agglutination reactions obtained with these sera in presence of emulsions of the Type bacilli.

The results of this titration of agglutinins show that:

(a) The *agglutinin* titre is no index of the *antitoxic* value of the serum: this of course is what would naturally be expected.

(b) Even when sera of high agglutinin titre are prepared, the agglutination reactions remain *specific*.

(c) The stock Type cultures have remained true to Type for a period exceeding 12 months.

(d) The agglutinin response is quite as marked when *washed cultures* are used for immunisation as when *whole cultures* are used for this purpose.

*Thirdly, the influence which these various sera exhibited in stimulating phagocytosis of Type bacilli was made the subject of inquiry.*

(c) *Technique of phagocytic tests.*

1. Washed white corpuscles (human) were prepared exactly according to the method advised by Wright; but in place of using a leucocyte layer in making the tests, the deposit of whole blood obtained after the final centrifugalisation was well mixed, and the thick blood cream so obtained was employed.

2. Unwashed actively growing 24-hour cultures served for the bacillary suspension. In order to obtain a sufficiently active growth, these cultures were made in trypsinised broth standardised to be alkaline to  $\alpha$ -naphthol-phthalein but acid to phenol-phthalein, and enriched by addition of fresh tissue.

3. The reaction was carried out at 38° C. and incubation proceeded for 15 mins. The mixtures of the various reagents were made and incubated in small agglutination tubes instead of in capillary tubes. This permits of the reagents being well distributed by shaking.

4. Each serum to be examined was tested in a series of dilutions according to the method of Neufeld, and all the sera were fresh when tested.

5. Each mixture consisted of:

1/50 c.c. culture,

1/50 „ of serum—usually diluted,

2/50 „ of washed blood cream.

6. After incubation, films were made in the ordinary way, fixed for two minutes in a mixture of equal parts of saturated aqueous solution of  $\text{HgCl}_2$  and absolute alcohol, and were stained for two minutes with 1/10 carbolfuchsin.

7. Examination of 100 consecutive leucocytes in each preparation was then made. I employed at first a 1/12 objective with a No. 4 ocular for making the counts, but subsequently found that, owing to the size of the bacillus, this could be done quite satisfactorily with 1/6 objective and a No. 4 ocular.

8. The method of Neufeld was adopted, as group relationship between the bacilli might be encountered. Dilution of the serum would to some extent overcome this difficulty.

9. No attempt was made to count the number of organisms taken up by each leucocyte; but, in order to obtain a relative figure, the leucocytes which contained considerable numbers of bacilli were regarded as positive cells, while those containing no bacilli, or only one or two, were considered negative.

Thus, in Table VII and subsequent Tables, the figures given represent the percentage of leucocytes in each preparation which took up the bacilli with avidity. It was remarkable how rarely one met with a cell containing few organisms; the leucocytes were either filled with bacilli or contained none. Difficulty of interpretation did not therefore arise. .

(d) *Results obtained in making phagocytic tests.*

The results of these phagocytosis experiments are shown in Table II.

Table VII. *The figures relate to the percentage of leucocytes taking up large numbers of bacilli.*

		Serum No. I = Type I toxin Dilutions of serum		
Culture used	No serum control	1/20	1/40	1/80
Type I	3	5	6	2
„ II	0	0	0	0
„ III	0	0	0	0
		Serum No. II = Type I 24-hr. whole culture		
Type I	x	85	92	84
„ II	x	10	6	1
„ III	x	77	14	15
		Serum No. III = Type I washed bacilli		
Type I	x	71	48	33
„ II	x	0	0	0
„ III	x	17	9	0
x = Not done.				

*Note.* In this and in subsequent tables relating to phagocytic experiments, the dilutions indicated at the head of each column give the dilution of serum originally made, so that in this instance, the actual dilutions in presence of which the test was carried out were 1/80, 1/160, and 1/320.

## VII. B.

Culture used	No serum control	Serum No. I' = Type II toxin		
		Dilutions of serum		
		1/20	1/40	1/80
Type I	1	0	2	2
„ II	0	0	0	0
„ III	0	0	0	0
Serum No. II' = Type II 24-hr. whole culture				
Type I	x	1	5	3
„ II	x	68	67	64
„ III	x	0	0	0
Serum No. III' = Type II washed bacilli				
Type I	x	5	1	4
„ II	x	50	31	37
„ III	x	0	0	0

## VII. C.

Culture used	No serum control	Serum No. I'' = Type III toxin		
		Dilutions of serum		
		1/20	1/40	1/80
Type I	0	0	2	1
„ II	0	0	0	0
„ III	2	2	0	0
Serum No. II'' = Type III 24-hr. whole culture				
Type I	x	87	89	85
„ II	x	28	16	5
„ III	x	99	98	100
Serum No. III'' = Type III washed bacilli				
Type I	x	2	2	1
„ II	x	4	1	0
„ III	x	44	26	4

*Note.* Experiments VII A and VII B were done together, using the same reagents, so that they are strictly comparable. My equipment did not permit of the simultaneous examination of all three, so that VII C was done on the following day.

The following points call for comment:

1. The *antitoxic* sera do not stimulate phagocytosis of the bacilli in presence of *whole culture*. It is to be noted, that the observations made with *antitoxic* sera serve as controls for the experiments with *anti-bacterial* sera.

2. The most active sera for stimulating phagocytosis are those prepared by inoculation of *whole culture*.

3. The sera prepared by inoculating *washed bacilli*, while giving an agglutinin titre as high as those prepared by inoculating *whole culture*, do not exhibit so high a phagocytic titre.

4. As regards the question of specificity, sera Nos. III, III', III'' (*i.e.* antisera to *washed bacilli*) exhibit this markedly in the above experiments, as also does No. II' (*i.e.* an antiserum to *whole culture* of Type II bacilli),

whereas Nos. II and II'', also *anti-whole* culture sera to Types I and III respectively, do not.

*Note.* Animal No. II' had been under immunisation for a shorter period than II and II''.

In view of the equivocal result obtained with sera Nos. II and II'', the experiment was repeated on the following day, but the dilutions of the sera were carried much further.

Serum No. III, owing to its having a demonstrable antitoxic content, 1 unit per c.c., was also included in this test, the results of which are shown in Table VIII.

Table VIII.

		Serum No. II = Type I 24-hr. whole culture				
		Dilutions of serum				
Cultures used	No serum control	1/50	1/100	1/200	1/400	1/800
Type I	0	89	63	50	4	0
„ III	0	5	2	0	0	0
		Serum No. II'' - Type III 24-hr. whole culture				
Type I	×	37	13	4	0	0
„ III	×	98	78	36	0	0
		Serum No. III = Type I washed bacilli				
Type I	×	24	14	2	0	0
„ III	×	1	0	0	0	0

*Note.* These experiments have been repeated many times and have given consistently specific results on each occasion. The experiment quoted (Table VII A and C) was *that one* of the series, which was most difficult to interpret.

I wish to call attention also to the discrepancy between the actual figures noted in Table VII, and those in Table VIII, in respect of the sera under examination; this appears to depend upon a variability of the anti-phagocytic properties of the cultures, or upon a variability of the phagocytic activity of the white cells from day to day, and renders futile any attempt that might be made to express these results in the form of an index of phagocytosis, unless a standard serum were made the basis of that index.

In order definitely to confirm the most important of the findings obtained in this series of investigations, viz. that *antitoxic* serum did not promote phagocytosis as did *anti-bacterial* serum, the experiment shown in Table IX was carried out.

Table IX.

		Serum No. I - Type I antitoxic		
		1/1	1/10	1/20
Type I		24	0	0
		Serum No. II = Type I 24-hr. whole culture		
Type I		79	82	84
		Serum No. I'' = Type III antitoxic		
Type III		34	0	2
		Serum No. II'' = Type III 24-hr. whole culture		
Type III		70	90	96

Normal serum controls 1/1 were included in the series, and gave substantially the same results as those obtained with 1/1 antitoxic serum.

In making this experiment, the technique already described was followed, but, to ensure that the employment of a 1/6 objective did not lead to avoidable error, the examination of the slides was made with a 1/12 semi-apochromatic lens with a No. 4 ocular, and care was taken critically to illuminate the preparation so that optimum conditions for microscopical examination were realised.

*Fourthly, the apparent anti-phagocytic property of whole culture was made the subject of investigation, and an attempt was made to determine whether toxin, as ordinarily prepared and stored, was also anti-phagocytic.*

(e) *Anti-phagocytic property of whole culture.*

Mechanism of the "anti-phagocytic property" of *whole culture*.

The results quoted in the previous Sub-section of this Report, suggest that the phagocytic activity of leucocytes in presence of anti-serum to *whole culture* may depend upon one or other of two things.

(i) That the serum prepared by inoculation of *whole culture* contains an antibody to an hypothetical aggressive antigen not present in *washed cultures*, and if present in filtrates, only in small quantity, or

(ii) That the *bacillary* substance, *per se*, is the antigen that determines the development of the property of stimulating phagocytosis in *anti-bacterial sera*.

It is not improbable that both factors, (i) and (ii), may be represented in the mechanism. By comparing Serum II with Serum III, II' with III', and II'' with III'', it is seen, that while the *anti-whole culture sera* are the more active in the presence of *whole culture*, yet the *anti-washed bacilli sera* are not altogether devoid of the power of inducing phagocytosis.

For the present then, leaving out the consideration of the question as to whether these antibodies which lead to the phagocytosis of tetanus bacilli are "stimulins," in the sense implied by Leishman, or "opsonins," in the sense implied by Wright, the point at issue was to determine if:

(a) *whole young culture* exhibited aggressive qualities not present in toxin obtained by filtration,

(b) these aggressive properties were specific to the serological Types of the bacilli, and could be countered by anti-bacterial serum prepared by inoculating *whole culture*.

The experiments quoted (Tables VII, VIII and IX, pp. 125-127) dispose of the question as to whether these aggressive properties can be neutralised by anti-bacterial (*whole culture*) serum.

To determine whether young *whole culture* did exhibit definite aggressive qualities, and at the same time to determine whether these were or were not specific to the serological Types, the following experiment was set up.

*Technique.*

Two centrifuge tubes of a 24-hour culture of Type I bacilli and two similar tubes of a 24-hour culture of Type III bacilli were centrifuged at high speed, in order to separate the bacilli from the products of their growth. The supernatant fluid from each tube was drawn off and kept separate. This procedure gave two tubes containing a deposit of Type I bacilli—tubes A and B—and two tubes containing a deposit of Type III bacilli—tubes C and D—and four tubes of supernatant fluid corresponding to these—namely A' and B', containing Type I supernatant fluids, and C' and D', containing Type III supernatant fluid.

The deposit in each of the four tubes was well shaken with saline and centrifuged. This procedure was repeated twice; after which the saline was finally drawn off with a pipette.

(1) To the deposit of washed Type I bacilli in A was added the supernatant fluid from a Type I culture—A'.

(2) To the deposit of washed Type I bacilli in B was added the supernatant fluid from a Type III culture—D'.

(3) To the deposit of washed Type III bacilli in C was added the supernatant fluid from a Type I culture—C'.

(4) To the deposit of washed Type III bacilli in D was added the supernatant fluid from a Type I culture—B'.

These four suspensions of bacilli were then exposed to washed white cells in presence of dilutions of Type I and Type III *anti-bacterial* sera. The results shown in Table X were obtained.

Table X.

Serum No. II=Type I 24-hr. whole culture				1/100	1/200
(a)	Type I whole culture	...	...	75	46
(b)	Type I bacilli + growth products of Type III	...	...	72	14
Serum No. II'' - Type III 24-hr. whole culture					
(c)	Type I whole culture	...	...	11	4
(d)	Type I bacilli + growth products of Type III	...	...	30	8
Serum No. II - Type I 24-hr. whole culture					
(e)	Type III whole culture	...	...	6	7
(f)	Type III bacilli + growth products of Type I	...	...	31	18
Serum No. II'' - Type III 24-hr. whole culture					
(g)	Type III whole culture	...	...	80	67
(h)	Type III bacilli + growth products of Type I	...	...	80	38

*Note.* The ordinary technique was followed in the above experiment, but the observations were made with a 1/12 objective and a No. 4 ocular, in order to exclude technical error as far as possible. In making the count, only those leucocytes containing no organisms were considered negative. This procedure had to be adopted in the present instance, as it was difficult to be sure that centrifugalisation had removed all the bacilli from the supernatant fluid.

Control experiments in which the supernatant fluids alone were exposed to the action of the *homologous* serum in presence of leucocytes showed that:

(a) When Type I supernatant fluid + Type I serum 1/100 + blood were incubated, 12 leucocytes were found to have taken up organisms.

(b) When Type III supernatant fluid + Type III 1/100 serum + blood were incubated, five leucocytes were found to contain organisms.

The real cause of the low counts obtained was, that there were so few bacilli present that many of the white cells never came into contact with bacilli, and the error therefore, due to the presence in these fluids of organisms that had not been deposited by centrifugalisation, was not very great.

Indeed, the error was more apparent than real, for there were so few bacilli present in these supernatant fluids that white cells containing more than two rods were seldom met with in the controls; while, in the above Table, the majority of the leucocytes considered as positive contained at least four or five bacilli.

In Table X the following points call for comment.

(i) When bacilli, supernatant fluid and serum are all *homologous*, consistently high counts are obtained with both dilutions of the serum—(a) and (g).

(ii) When bacilli and supernatant fluids are both *heterologous* to the serum, consistently low counts are registered with both dilutions—(c) and (e).

(iii) Where the bacilli are *homologous* to the serum and the growth products are *heterologous* thereto, a high count is obtained in presence of the higher concentration of the serum; but—as compared with the control, where *homologues* only are present in the mixtures—the lower concentration of the serum shows a relatively low phagocytosis—(b) and (h).

(iv) When the bacilli are *heterologous* to the serum and the growth products *homologous* to it, a relatively high count—as compared with the control in which both bacilli and growth products are *heterologous*—is obtained with the higher concentration of the serum—(d) and (f).

(v) The results obtained with *whole* cultures confirm that the reactions are specific to the Types.

From these observations the following deductions seem permissible.

(a) That the reaction to inoculation of *bacillary* substance alone, results in the development of antibodies which are specific to each Type and induce phagocytosis.

(b) From (iii) it may be deduced that while aggressive substances appear to exist in young cultures, these can, to a considerable extent, be neutralised by an *heterologous anti-bacterial* serum; it is only when the *heterologous serum* is diluted, that the specific aggressive quality becomes manifest.

(c) From (iv) the same deduction may be made. For it is seen that leucocytes, even when brought into contact with bacilli which are not *homologous* to the serum present in the mixture, show considerable phagocytic activity, provided that the bacterial products in which the bacilli are sus-

pended *are homologous* to the serum. This activity is naturally most marked in presence of the higher concentrations of serum, and appears to be specific in respect of the products of the various serological Types.

I do not wish to lay over much stress on this Section of the work knowing well the errors, both technical and subjective, that are liable to be introduced in such experiments.

The tests have been repeated and consistent results have been obtained so far as the method allows. Nevertheless, the evidence is by no means unequivocal. I submit, however, that it is highly suggestive, and indicates that there are at least three antigenic factors in cultures of *B. tetani*.

(i) The *spasm-producing toxin*, which is filtrable, and is non-specific in relation to the Types.

(ii) The *bacillary* substance, which is definitely specific to these Types.

(iii) A third *antigen*, which has anti-phagocytic properties, and appears to be specific to the Types.

These findings corroborate those which were obtained when comparative tests were made with *antitoxic*, *anti-whole-culture* and *anti-washed-bacilli* sera; indeed, the deductions which may be made from the one series of experiments are in complete agreement with those that may be made from the other.

*Finally, the question of whether toxin, as ordinarily prepared by filtration, exhibits anti-phagocytic properties, was made the subject of investigation.*

(f) *Examination of toxin to determine whether it is leucotoxic.*

In inquiring into whether tetanus toxin was leucotoxic, I made use of two methods of investigation.

The first series of tests was carried out thus:

Phagocytic tests were made with serum 'No. III'', which was prepared by inoculation of *washed* Type III bacilli—agglutinin titre 1/3200, antitoxic value < 0.5 unit per c.c., phagocytic titre low. The serum was diluted and exposed to various mixtures of reagents prepared thus:

Five tubes of a 24-hour culture of Type III bacilli were centrifugalised, and the deposit in each was retained. This deposit was washed by adding saline and again centrifugalised, the process being repeated twice. The supernatant saline, after the final centrifugalisation, was pipetted off so that a deposit of washed Type III bacilli was obtained in each of the five tubes—*a*, *b*, *c*, *d* and *e*.

(1) To deposit in (*a*) saline was added to the original volume.

(2) To deposit in (*b*) was added supernatant fluid obtained by centrifuging a 48-hour culture of Type III bacilli.

(3) To deposit in (*c*) was added the supernatant fluid from a 10-day culture of Type III bacilli.

(4) To deposit in (*d*) was added the toxin obtained by filtration of a 48-hour culture of Type III bacilli. This toxin had been stored in the ice-chest for three months before the test was made.



(5) To deposit in (e) was added the toxin obtained by filtration of a 10-day culture of Type III bacilli prepared six months before the test was made.

Phagocytic tests were then set up using each of these suspensions as the bacillary emulsions to be phagocytosed. The results shown in Table XI were obtained.

Table XI.

Serum No. III'' = Type III washed bacilli

Washed bacilli Type III + saline ...	1/20	1/40	1/80
... ..	46	18	9
„ „ + supernatant fluid of 2-day culture	10	2	0
„ „ + „ „ 10 „	3	0	0
„ „ + toxin from 2 „	12	2	1
„ „ + „ „ 10 „	39	13	2

These results show, that both the supernatant fluid and the toxin from the 2-day culture which was used, are definitely anti-phagocytic; while the toxin from the 10-day culture which was included in the test is not so.

It is to be noted that the 2-day toxin was not more lethal for mice than was the 10-day toxin.

With reference to the loss of anti-phagocytic potency in the case of the 10-day toxin, it is probable, that the prolonged storage rather than the prolonged incubation or the filtration, has been responsible for the decline; for, in respect of its anti-phagocytic power, it compares unfavourably both with the 2-day toxin and with the 10-day supernatant fluid.

As the phagocytic potency of Serum III''—*anti-washed-bacillary*—was known to be low, the experiment was repeated, using Serum No. II''—*anti-whole-culture*—the phagocytic potency of which was known to be high, and which also exhibited a demonstrable antitoxic titre. Using this serum the following results were obtained.

Table XII.

Serum No. II'' = Type III 24-hr. whole culture

Washed bacilli Type III + saline ...	1/50	1/100	1/200
... ..	27	73	61
„ „ + supernatant fluid of 2-day culture	55	45	41
„ „ + „ „ 10 „	64	54	45
„ „ + toxin from 2-day culture ...	47	44	28
„ „ + „ „ 10 „ ...	66	65	60

The results shown in Tables XI and XII call for the following comments.

(a) The figures in Table XI leave no room for doubt that both supernatant fluids resulting from centrifugalisation of cultures of *B. tetani*, and certain toxins obtained by filtration, are anti-phagocytic. The questions of whether filtration reduces this leucotoxic factor, whether it stands storage, and at what period of growth it is present in largest quantity, or in most active condition, are not answered by the experiment quoted. In this instance—Table XI—it is possible, that owing to the low phagocytic potency of the

serum, and the absence of antitoxic power, a trace of the anti-phagocytic property in the fluids might suffice to obliterate phagocytosis. The conditions of the experiment then do not permit of quantitative results being obtained except under exceptional circumstances, *e.g.* with the 10-day toxin after prolonged storage.

(b) The results shown in Table XII, beyond demonstrating that the anti-phagocytic activity both of toxin and of fluid obtained by centrifugalisation are neutralised by *anti-whole-culture* serum, fail to answer the questions raised.

In the second series of experiments an attempt was made to solve certain of these problems, by making indirect experiments, in which an indifferent substance was employed as the body to be taken up by the phagocytes.

It is to be noted, that the results set forth in Tables XIII and XIV are given with every reservation, for subsequent inquiry may lead either to very different results being obtained, or very different interpretations of the facts being made; for the technical difficulties, both subjective and objective, that have to be overcome, are such, that a series of crucial experiments cannot be carried out.

#### *Technique.*

The first technical difficulty that arose was the determination of a suitable indifferent substance for phagocytosis. After many trials had been made, I ultimately decided to use a boiled culture of *Staphylococcus albus* for this purpose. The staphylococci, after boiling, were readily taken up by the leucocytes in presence of fresh normal rabbit serum.

Mixtures consisting of 1/50 c.c. of staphylococcus suspension, 1/50 c.c. of the serum under examination, 1/50 c.c. of the toxin to be investigated and 3/50 c.c. of blood suspension were prepared in the same way as for previous experiments. These were incubated for 15 minutes, and preparations made and examined as before. In examining the slides a combination of 1/12 objective and No. 4 ocular was used throughout the series. The results shown in Table XIII were obtained.

Table XIII.

Dilution of serum = 1/2, *i.e.* 1/12 of the final mixtures.

Figures express percentage of leucocytes taking up many staphylococci.

				Serum diluted 1/2				
Toxin (undiluted)				1/50 c.c.	Staphylo. 1/50 c.c.	Blood 3/50 c.c.	Normal serum 1/50 c.c.	Type II antitoxin 1/50 c.c.
No toxin—saline only				...	...	...	69	×
Toxin from 2-day growth Type II				..	..	..	7	..
..	10	..	.. II	..	..	..	42	..
..	2	..	.. III	..	..	..	31	..
..	10	..	.. III	..	..	..	68	..

The figures indicate that *antitoxic* serum does neutralise the *anti-phagocytic* property of toxin. It may here be noted again, that the toxin from the 2-day culture is more anti-phagocytic than the toxin from the 10-day culture. As to whether the reaction is or is not specific in relation to the Types, the results obtained are equivocal; for the anti-phagocytic power of both the Type III toxins used in the test appears to be less than that of the Type II toxins.

The converse experiment was therefore carried out using a 1/5 dilution of Type III *antitoxic* serum and the results shown in Table XIV were obtained.

Table XIV.

Dilution of serum = 1/5, i.e. 1/30 of final mixtures.

Figures express percentage of leucocytes taking up many staphylococci

Toxin (undiluted)	Serum diluted 1/5			Normal serum 1/50 c.c.	Type III antitoxin 1/50 c.c.
	1/50 c.c.	Staphylo. 1/50 c.c.	Blood 3/50 c.c.		
No toxin—saline only	...	...	...	86	×
Toxin from 2-day growth Type III	..	..	..	10	63
.. 10 .. " III	..	..	..	41	83
.. 2 .. " II	..	..	..	22	34
.. 10 .. " II	..	..	..	38	83

*Note.* Experiments XIII and XIV were done on different days so that they are not strictly comparable.

Here again the *anti-phagocytic* property of toxin is apparent, and again, the toxin of the 2-day growth exhibits greater anti-phagocytic power than does that of the 10-day growth. Specificity appears to be fairly definite in this instance, at least so far as the toxin from 2-day growth is concerned. Thus, in the case of the *homologous* mixture, the number of active leucocytes is 63, as contrasted with 10 in the normal serum control. While, in the case of the *heterologous* mixture, it is 34 as compared with 22 for the corresponding control.

The results of these experiments are quite definite in respect of the following points.

- (i) Toxin is anti-phagocytic.
- (ii) The anti-phagocytic property of toxin can be neutralised by antitoxin.
- (iii) If a specific relationship between toxin and antitoxin exist in this connection, the relationship is *quantitative*—the experiments quoted suggest such relationship.

In view of the variability of the anti-phagocytic power of the cultures and their products, a crucial experiment is difficult to perform. Although certain of the results then do suggest such a specific relationship, they fail to demonstrate that relationship unequivocally.

## RÉSUMÉ OF SECTION IV.

(I) *Antitoxic sera* do not stimulate phagocytosis of tetanus bacilli.

(II) *Anti-bacterial sera*, prepared by inoculation of *whole* culture, markedly stimulate phagocytosis; and the relationship between serum and organism is specific to the serological Types.

(III) Although *anti-bacterial sera*, prepared by inoculation of *washed* cultures, are active from the standpoint of agglutination, they are not so active in stimulating phagocytosis as are sera obtained by inoculation of *whole* culture.

(IV) The agglutinin titre, the antitoxic titre, and the phagocytic titre of the sera, are independent one of another.

(V) Tetanus toxin is apparently leucotoxic, but all specimens of toxin are not equally leucotoxic.

(VI) This leucotoxic factor can be neutralised by antitoxin; and the experiments quoted suggest that the neutralisation, by antitoxin, of this leucotoxic factor in toxin is specific in relation to the bacillary Types.

## SECTION V.

## INVESTIGATION OF MECHANISM OF INFECTION IN TETANUS.

## INTRODUCTION.

Section IV (pp. 120-135) of this Report shows definitely, that, apart from agglutinins, antibodies specific to the serological Types of *B. tetani* can be evoked by injecting *whole* cultures of that organism into animals. These findings are of considerable significance, but, unless it can be demonstrated *in vivo*, that anti-bacterial immunity plays some part in the prevention or cure of tetanus, their interest is largely academic.

In the first place, it must be clearly understood, that tetanus is due to *the growth* of *B. tetani* within, or in close proximity to, the tissues; and, that intoxication results only when sufficient growth of the organism has occurred. This emphasis of a palpable platitude is necessary, for, in the past, the attention of investigators has been so directed to the mechanism of intoxication in tetanus, that the conditions, which in nature must necessarily precede that intoxication, have been largely overlooked.

In this Section of the Report it is proposed to inquire into the mechanism of *Tetanus infection*, and to determine, if possible, whether the prophylaxis of the disease could be improved in any direction. This really involves the consideration of

- (i) Surgical measures.
- (ii) Concomitant infections, and how to deal with them.
- (iii) The possible value of anti-bacterial sera.

Before these problems could be examined, much preliminary work had to be done in order to elaborate a satisfactory technique for infecting the animals which would be used in the investigation. This presents considerable difficulty, for infection with *B. tetani*, and indeed, infection with any of the pathogenic anaërobes, is, at least in the early stage, saprophytic rather than parasitic in quality. Thus, it has long been known that inoculation of *washed* and *heated* tetanus spores does not frequently lead to the development of the disease, and it is only when a certain amount of tissue destruction has occurred that infection will "take." The same is true of infection with *Vibrio septique*; Besson has shown that spores of this organism may be injected into animals with no ill-effect; if, however, these spores be suspended in a fluid which has leucotoxic properties, infection invariably occurs. Emulsions of *B. Welchii* deprived of their growth products have also been injected without leading to infection, but when injections of the emulsions together with their growth products are made "trauma"—really due to presence of toxin—occurs, leading to the development of the organism.

These considerations have a direct bearing upon the causation of tetanus; for, concomitant infection with any pathogenic organism, by exerting a tissue-debilitating influence, may permit of the development of *B. tetani*. In natural infection of wounds with anaërobes, one cannot consider one infection alone, as every organism in the flora of the wound may exert an influence, stimulant or deterrent, upon the development of any one type of organism present. Wounds probably pass through a cycle of infections; certain aërobes and then certain of the more rapidly growing anaërobes appear in the first phases, to be followed subsequently by the more slowly developing bacteria including *B. tetani*. It must not be assumed, however, that all wounds will pass through the same cycle; for it will depend partly upon the relative numbers of the various organisms present in the infecting material, and partly upon peculiarities of the species or individual exposed to infection, which of the organisms present in the *inoculum* will develop first, and what sequence they will thereafter follow.

In the causation of tetanus the primary *necessary* factor is a certain degree of tissue destruction. In the nidus so formed, organisms grow, and among the earliest usually to appear are certain of the bacilli responsible for the causation of gas gangrene.

These organisms grow rapidly—especially *B. Welchii*--developing growth products which exert a necrotic action upon the tissues. This permits of the further growth of *B. tetani* and of other bacteria of specific infections. A vicious cycle is thus established, and the fact must not be lost sight of, that before even these adjuvant infections occur, it is essential that there should be tissue debility caused by direct trauma to the part, or trauma to important structures in relation thereto.

Therefore, the most important prophylactic measure which can be applied

for the limitation of anaërobic infections is, *adequate and enlightened surgical interference*, performed at the earliest possible moment after reception of the injury. Therefore wide resection of wounds, by removing devitalised tissue, prevents the establishment of this vicious circle.

This question, which is of paramount importance in the experimental work dealt with in this Section of the present communication, is more fully considered in Section VI, pp. 172–195.

These considerations prompted the following questions.

- (a) DOES *B. TETANI*, IN ADDITION TO ELABORATING A SPASM-PRODUCING SUBSTANCE, ALSO GIVE RISE TO A TISSUE DEBILITATING POISON?

Section No. IV of this Report indicates that *B. tetani* gives rise to tissue-debilitating poisons, for the products of its growth are definitely leucotoxic. In order further to inquire into this problem the following *in vivo* experiment was carried out.

The M.L.D. for rats, of a tetanus toxin obtained from a 10-day growth of bacilli—stored for three months in the ice-chest before use—was first determined. The fraction of the M.L.D. which would invariably give rise to local tetanus but would not cause death was next determined. Mixtures of this quantity of toxin with 2000 million washed but unheated tetanus bacilli (obtained by successive centrifugalisation of rapidly growing cultures suspended in saline) were then made.

(a) Mixture consisting of “local tetanus producing dose” of toxin + Type I bacilli 2000 million.

(b) Mixture consisting of “local tetanus producing dose” of toxin + Type II bacilli 2000 million.

(c) Mixture consisting of “local tetanus producing dose” of toxin + Type III bacilli 2000 million.

(d) Saline + Type I bacilli 2000 million.

Four rats were then inoculated, each with one of the above mixtures. All excepting (d), which remained well, developed local tetanus between the third and sixth days after inoculation; the spasm continued for 7–10 days and thereafter the animals recovered.

This experiment, while failing to prove, or even to suggest, that *B. tetani* does not develop a tissue debilitating substance, shows that a dose of toxin sufficient to cause a temporary disturbance of the anterior horn cells, may not necessarily exhibit, *locally*, qualities sufficiently aggressive to set up infection.

The *spasm-producing* element of the toxin then is not necessarily a determining factor in the mechanism of *infection*.

I have attempted to carry out the converse experiment but have so far met with no success, *i.e.* I have not been able to obtain a culture possessing sufficient aggressive quality, and at the same time of sufficiently

low "spasmin" content, to permit of a differentiation between local debilitating effect with subsequent *infection*, and immediate disturbance due to central nervous system *intoxication*.

(b) AS TISSUE DEBILITATING INFLUENCES CERTAINLY ASSIST IN THE DEVELOPMENT OF TETANUS INFECTION, IS THERE ANY PARTICULAR DEBILITATING INFLUENCE THAT IS OF SPECIAL SIGNIFICANCE IN THIS CONNECTION?

Apart from purely physical considerations such as situation and degree of original trauma, coincident involvement of particular structures, muscle, nerves, vessels, etc., the necrotising influence of the products of certain organisms may play an important part in determining the genesis and evolution of tetanus infection.

That concomitant infection with other organisms might exert such an influence upon the growth of *B. tetani* in wounds, and that this influence might vary with the nature of such concomitant infection, was foreseen by Vaillard and Vincent. The question became insistent when the importance of gas gangrene infections was appreciated in this connection. The following experiments were undertaken with a view to inquiring into the relationship which infection of wounds with *B. Welchii* and *Vibrio septique* might bear to the causation of tetanus.

A preliminary series of experiments was undertaken.

(1) To determine approximately how many washed and heated spores of *B. tetani* could be injected intramuscularly into the guinea-pig without causing the disease.

(2) To determine what dosage of the toxins of *B. Welchii* and *Vibrio septique*, respectively, produced tetanus when injected along with a constant number of spores of *B. tetani*.

In the experiments with *B. Welchii* it was found that 0.2 c.c. of a toxin (which in a dose of 1 c.c. killed one out of three guinea-pigs of 250 grammes weight) sufficed to cause enough local disturbance to determine invariably the onset of tetanus, when injected along with 1000 million spores.

A. INFLUENCE OF TOXIN OF *B. WELCHII* ON THE DEVELOPMENT OF TETANUS SPORES *IN VIVO*.

*Experiment I.*

Guinea-pig I was inoculated in the right gastrocnemius with 1000 million spores Type I (U.S.A.) in 1 c.c. saline. For the first five days the animal was well; on the sixth and seventh days it limped slightly (? local tetanus); on the eighth day there was no obvious limp, and for the remaining six days during which the animal was under observation it remained well.

Guinea-pig II was inoculated with 1000 million spores Type I plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.). The following day there were signs of local tetanus; on the second day there was definite local tetanus; and on the third day the animal died from generalised tetanus.

Guinea-pig III also received 1000 million spores Type I plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.) in the right gastrocnemius. One hour later 1400 units of *B. Welchii* antitoxin were injected into the left gastrocnemius. The animal showed no signs of tetanus, and remained well during the period in which it was under observation.

### *Experiment II.*

Guinea-pig I A was inoculated in the right gastrocnemius with 1000 million Type II tetanus spores in 1 c.c. saline. It remained well for the first five days; on the sixth there was a slight limp (? local tetanus); on the seventh day there was definite local tetanus; on the eighth general tetanus; then the animal was killed.

Guinea-pig II A was inoculated with 1000 million Type II tetanus spores, plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.). On the first day the animal appeared to be well; on the second it was moribund, and was then killed.

Guinea-pig III A received 1000 million Type II tetanus spores plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.) in the right gastrocnemius. Two hours later 1400 units of *B. Welchii* antitoxin were injected into the left gastrocnemius. For the first five days the animal was well; on the sixth and seventh days it slightly limped (? local tetanus); on the eighth day the limp was less marked; and on the ninth day the animal was well, and it remained well during the period in which it was under observation.

### *Experiment III.*

Guinea-pig I B was inoculated with 1000 million Type III tetanus spores in 1 c.c. saline, in the right gastrocnemius. Guinea-pig II B received 1000 million spores Type III, plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.). Guinea-pig III B was inoculated in the right gastrocnemius with 1000 million spores Type III, plus 0.2 c.c. *B. Welchii* toxin (volume 1 c.c.), and received 1400 units *B. Welchii* antitoxin in the left gastrocnemius. Guinea-pig I B remained well; Guinea-pig II B died of tetanus on the third day; and Guinea-pig III B, beyond exhibiting slight stiffness of the inoculated limb from the sixth to the fourteenth day after inoculation, remained well.

The deductions to be drawn from these three experiments are self-evident. They indicate that an antitoxin for *B. Welchii* and tetanus antitoxin should be employed together for the routine serum prophylaxis of tetanus. The evidence is unequivocal that the antitoxin of *B. Welchii*, in addition to neutralising its toxin, has the advantage that it completely protects (in the case of the guinea-pig) tissue which has been exposed to the devitalising effect of that toxin against the development of tetanus spores. It is highly probable that the same protecting value would be found in the case of man.

Attention is called to the death of the control animal I A in Experiment II. The death of this animal, while in no way detracting from the validity of the experiments or of the deductions to be drawn from them, indicates that the development of tetanus depends upon a number of different factors. It is not improbable that, in this instance, a certain degree of trauma when the inoculation was made, was responsible for the development of the infection. It is to be specially noted, however, that while the test animal II A died on the second day after inoculation, the control animal did not die until the eighth day. Moreover the test animal died of acute, almost fulminating tetanus, while the course of the disease in the control animal was much less acute. Experiment II is, therefore, exceptionally instructive; for, while it



shows that the danger of infection with *B. tetani*, and notably the early development of the disease owing to "symbiosis" with *B. Welchii*, could be considerably mitigated—if not completely eliminated—by the use of a combined serum for prophylaxis, it also called attention to the fact, that while such a reagent would probably be of great value, surgical measures should not be less carefully applied because of its introduction.

I call attention to this because, if a combined prophylactic serum were to be introduced, it might lead to the development of a feeling of false security, and too optimistic a view might be taken regarding the probable value of such a combined serum. As a natural sequence its unmerited condemnation would ensue, if the results obtained did not completely bear out expectations.

#### B. INFLUENCE OF THE TOXIN OF *VIBRION SEPTIQUE* ON THE DEVELOPMENT OF TETANUS SPORES *IN VIVO*.

*Vibrion septique* is another of the organisms which are commonly found in wounds, and develops diffusible toxic products. While it is not known whether the toxin of this organism is a particularly active necrotising substance or only has marked oedema-producing properties, it seemed of importance, nevertheless, to determine the possible influence which it might exert upon the development of tetanus.

As the properties of this toxin are at present less well defined than are those of the toxin of *B. Welchii*, the experiments to be described in this section cannot be so clearly interpreted as are Experiments I, II and III, pp. 138–139. This difficulty is enhanced by the fact that, at the time of writing, specific *Vibrion septique* antitoxic serum was not available, so that protection experiments could not be undertaken.

I here wish to record my thanks to Miss Robertson of the Lister Institute of Preventive Medicine for placing at my disposal a quantity of the toxin of *Vibrion septique*.

The methods employed and the results obtained in making this investigation were as follows:

#### *Experiment IV.*

Guinea-pig A was inoculated in the right gastrocnemius with 0.25 c.c. *Vibrion septique* toxin plus 0.75 c.c. saline. On the first day there was slight oedema and stiffness of the leg. On the second day the oedema was palpable, but not extensive; there was still stiffness of leg. On the third day the oedema was almost gone and the leg less stiff. On the fourth day the animal had almost recovered, and on the fifth day it was well, and remained so during the period of the experiment.

Guinea-pig B was inoculated with 0.25 c.c. *Vibrion septique* toxin, plus 0.25 c.c. saline plus 1000 million tetanus spores Type I. On the first day there was slight oedema and stiffness of leg. On the second day oedema palpable but not extensive; and there was still stiffness of leg. On the third day the animal was recovering, and on the fourth it had almost recovered. On the fifth day it was well, and remained so.

Although the oedema resulting from the injection of 0.25 c.c. of this toxin was quite as marked as, if not more marked than, that produced by 0.2 c.c. of the toxin of *B. Welchii*, it is remarkable that tetanus did not develop. I therefore decided to make the test much more stringent, employing 1 c.c. of the toxin plus 1000 million tetanus spores. The result of an experiment employing this technique is shown below.

#### *Experiment V.*

Animal C was inoculated in the right gastrocnemius with 1 c.c. *Vibrio septique* toxin plus 0.5 c.c. saline. On the first day there was marked oedema over the whole of the injected limb; on the second the oedema was more marked and extending over the abdominal wall, soft on palpation. The next day the oedema was reduced and the animal lively; limb stiff. On the fourth day the oedema was disappearing; leg still stiff. On the fifth day the animal had recovered, and remained well till the conclusion of the experiment.

Animal D received 1 c.c. *Vibrio septique* toxin plus 1000 million Type I tetanus spores. On the first day there was marked oedema over the whole of the injected limb; on the second, oedema as in Animal C. On the third day the oedema was reduced and the animal lively, but limb stiff. Fourth day, oedema almost gone; leg still stiff. On the fifth day the animal was well, and remained so.

A slight stiffness of the limb remained in both animals until the completion of the experiment, which was considered to have occurred on the fourteenth day. The animals have remained well, and the stiffness is slowly disappearing. This experiment is striking, in that, although the oedema produced in the animals was much more marked than that resulting from the injection of 0.2 c.c. of the toxin of *B. Welchii* employed in the previous series of experiments, no development of tetanus occurred.

This result was scarcely expected, and the experiment was therefore repeated.

#### *Experiment VI.*

Animal E was inoculated with 1000 million Type I tetanus spores suspended in 1.5 c.c. of saline. Animal F with 1 c.c. of *Vibrio septique* toxin plus 1000 million Type I tetanus spores. These animals both remained well, so corroborating the findings indicated in Experiment V.

As the disturbance produced by *Vibrio septique* toxin—oedema production—was so pronounced, it seemed remarkable that tetanus did not develop. The experiment was repeated a second time, using spores of a representative Type III tetanus bacillus. The findings of this experiment are as follows:

#### *Experiment VII.*

Animal G was inoculated in the right gastrocnemius with 1000 million Type III tetanus spores suspended in 1.5 c.c. saline. It remained well. Animal H was inoculated with 1000 million Type III tetanus spores plus 1 c.c. *Vibrio septique* (volume 1.5 c.c.). There was marked oedema on the first day, but the animal was active. On the second day there was general tetanus, and the animal was killed.

As specific antitoxin for *Vibrio septique* was not available, it was decided not to complete the present series of experiments using Type II spores; for the results shown in Experiment VII indicate that *Vibrio septique* may, like *B. Welchii*, play an ancillary part in the causation of tetanus.

The negative results obtained on three occasions when Type I spores were used indicate, however, that the toxin of *Vibrio septique* is probably a less constant factor in stimulating the growth of *B. tetani* in the tissues, than is that of *B. Welchii*. In view, however, of its frequent occurrence, and in view of the fact that it sporulates more readily than does *B. Welchii*, its capacity for doing harm is possibly extended over a more prolonged period than is the case with *B. Welchii*. It would seem advisable therefore, that antibodies to the toxin of *Vibrio septique* should also be included in serum used for the prophylaxis of tetanus.

CONCLUSIONS FROM THIS SERIES OF EXPERIMENTS, pp. 138-142.

1. There is good ground for believing that the ancillary part played by *B. Welchii* in the causation of tetanus is clearly defined. The capacity of this organism for doing harm in the connection under consideration can be almost eliminated by the use of the antitoxin for *B. Welchii*.

2. The capacity of the toxin of *Vibrio septique* for stimulating the growth of tetanus spores *in vivo* is more variable than is that of *B. Welchii*. Experiment VII indicates, however, that it too may play a part in the causation of tetanus.

3. It follows from conclusions 1 and 2 that antibodies to the toxins of *B. tetani*, *B. Welchii*, and *Vibrio septique* should be included in all serum employed for the prophylaxis of tetanus.

4. While such a polyvalent serum promises to reduce still further the incidence of tetanus, we may not assume that it would absolutely eliminate that disease, for infections other than those dealt with in this communication may also play a part in stimulating the growth of *B. tetani* in wounds.

(c) AS CONCOMITANT INFECTION WITH CERTAIN ORGANISMS STIMULATES THE GROWTH OF SPORES OF *B. TETANI* IN TISSUE, IS IT NOT POSSIBLE THAT THE CONVERSE MAY BE EQUALLY TRUE? AT LEAST, MAY NOT SOME CONCOMITANT INFECTIONS REDUCE THE TOXOGENIC CAPACITY OF *B. TETANI* UNDER CERTAIN CIRCUMSTANCES WHICH ARE AT PRESENT UNKNOWN?

The facts dealt with in this Sub-section of the Report were obtained, not in making inquiry into the above question, but in conducting an investigation with another object in view. This problem is complementary to (b), p. 138. In examining wound exudates from men showing no evidence of tetanus, Miss Cayley obtained three consecutive cultures, which failed to develop toxin when tested by the ordinary methods, but nevertheless contained bacilli in large numbers having the morphological characters of *B. tetani* and, on investigation by the method described in a previous com-



Absorption of agglutinin tests were then carried out again using the standard technique, and results shown in Table XVI were obtained.

Table XVI A.

*Absorption of Type I Serum by Cultures C 5, C 14, C 15.*

Culture	Unabsorbed serum				Absorbed serum							
	Agglutination				Homologous bacillus added				Test bacillus added			
	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800
C 5	+	+	+	-	+	-	-	-	-	-	-	-
C 14	+	+	+	-	+	-	-	-	-	-	-	-
C 15	+	+	+	-	+	-	-	-	-	-	-	-
Control	+	+	+	-	-	-	-	-	-	-	-	-

Table XVI B.

*Absorption of Type II Serum by Cultures C 5, C 14, C 15.*

Culture	Unabsorbed serum				Absorbed serum							
	Agglutination				Homologous bacillus added				Test bacillus added			
	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800
C 5	-	-	-	-	+	+	+	+	-	-	-	-
C 14	-	-	-	-	+	+	+	+	-	-	-	-
C 15	-	-	-	-	+	+	+	+	-	-	-	-
Control	+	+	+	+	-	-	-	-	-	-	-	-

Table XVI C.

*Absorption of Type III Serum by Cultures C 5, C 14, C 15.*

Culture	Unabsorbed serum				Absorbed serum							
	Agglutination				Homologous bacillus added				Test bacillus added			
	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800	1/100	1/200	1/400	1/800
C 5	-	-	-	-	+	+	+	+	-	-	-	-
C 14	-	-	-	-	+	+	+	+	-	-	-	-
C 15	-	-	-	-	+	+	+	+	-	-	-	-
Control	+	+	+	+	-	-	-	-	-	-	-	-

The findings shown in Tables XV and XVI indicate that the three cultures under consideration were Type I tetanus bacilli, and, in view of their failure to produce toxin when tested in the ordinary way, inquiry had to be made to determine whether:

(a) They were intrinsically atoxic.

(b) The circumstances of their growth reduced their toxogenic capacity.

D. Animal experiments were therefore undertaken to investigate these points.

(1) In the first place sub-cultures into ordinary peptone broth were made from the original meat cultures, and were incubated anaërobically for six days.

Microscopical examination of the growths was then made to determine the

presence of organisms bearing spherical terminal spores. A massive inoculation—0.5 c.c.—of these growths was made intramuscularly into each of three rats. All the animals remained well, so confirming the original experiment. The experiment was repeated with mice and the same result was obtained.

(2) As it seemed possible that the tetanus bacilli might have been introduced into the enriching medium through technical error, sub-cultures were made in broth from the tubes containing growths of C5, C14 and C15 in the enriching medium. After 10 days 0.5 c.c. of each of these sub-cultures was inoculated into mice.

Notwithstanding these massive inoculations two of the mice remained well. That inoculated with C14 died from tetanus on the 5th day. It seemed improbable then, that the tetanus bacilli had been introduced into the enriching medium during any of the various processes of its preparation. In order further to ensure that the medium was not a source of error, sub-cultures from the original meat tubes were made in three different batches of enriching medium, and agglutination tests were repeated with the cultures so obtained. All gave consistent results.

*Note.* Before any batch of enriching medium is used in the laboratory, it is examined by cultural methods to ensure sterility, and where animal experiments are to be performed with growths of organisms in this medium, it is inoculated into mice to ascertain that the medium itself does not exhibit toxic properties.

(3) The following experiments were then set up to determine, if possible, whether the tetanus bacilli in C5, C14 and C15 were themselves atoxic, or whether their toxogenic capacity was interfered with by concomitant growth of other bacteria present in the cultures.

(i) Four tubes of meat broth, *a*, *b*, *c*, and *d*, were each inoculated with an equal quantity of the same culture of Type I bacilli—the U.S.A. standard culture which was known to be toxic.

Into Tube " <i>b</i> "	was then seeded	some of the original meat culture of	C5.
" " <i>c</i> "	"	"	" C14.
" " <i>d</i> "	"	"	" C15.

Tube "*a*," which was inoculated with the standard U.S.A. culture only, served as a control.

All four tubes were incubated for eight days, and then 0.25 c.c. of each culture—again a massive inoculation for mice—was injected subcutaneously into mice.

The mouse inoculated with "*a*" died from tetanus within 24 hours; that inoculated with "*b*" showed tetanic symptoms from the 3rd to the 8th day, but eventually recovered; that inoculated with "*c*" remained well; while the animal which received "*d*" developed tetanus only on the 5th day and died from the disease on the 6th.

(ii) The experiment was repeated but the tubes in this instance were incubated for 10 days.

The animal inoculated with "a" died within 24 hours; that with "b" showed tetanic symptoms from which the animal ultimately recovered—the onset of the condition occurring six days after inoculation. The animals inoculated with "c" and "d" remained well.

(iii) Exactly similar experiments were then carried out using cultures from mixed *inocula* of Type II and Type III bacilli and C 5, C 14 and C 15. The cultures were incubated for eight days and inoculated as in (i) and (ii). In neither case, however, did any of the growths prove atoxic.

(iv) Experiment (i) was repeated a second time and gave unequivocal confirmation of the previous findings.

(v) As experiments (i) and (ii) of this Sub-section indicated that the toxic qualities of mixed cultures might vary from day to day I made four sub-cultures each of C 5, C 14 and C 15 for inoculation on different days.

One series of tests was made after four, one after six, one after eight, and one after ten days' incubation. The *inocula* consisted of 0.5 c.c. in each instance, and the animals used were rats. All the animals in this series of experiments survived, and none showed evidence of tetanus.

(vi) The experiments described in (i), (ii) and (iv) indicate that coincident growth of other organisms may prevent the development of tetanus toxin in cultures.

In order finally to dispose of the question, sub-cultures from the original meat tubes of C 5, C 14 and C 15, were made in the enriching medium, and the growths so obtained were centrifugalised, washed, and heated, as for the preparation of suspensions for agglutination. In doing this, special precautions were taken to prevent the occurrence of contamination. A specimen of each of the suspensions so obtained was retained for animal inoculation, and the bulk of each suspension was agglutinated. Again all three cultures reacted well in the presence of Type I agglutinating serum.

The specimens removed from each suspension before agglutination were dealt with thus:

(a) 0.1 c.c. of suspension of C 5 was mixed with 1/4 M.L.D.—for mice—of *Welchii* toxin. The mixture, so prepared—total volume 0.2 c.c.—was injected subcutaneously into a mouse. The animal developed tetanus and died on the third day.

(b) 0.1 c.c. of suspension of C 14 + the same quantity of *Welchii* toxin was injected into a second mouse. The animal developed tetanus, and was killed on the second day after inoculation.

(c) An exactly similar experiment in which the suspension of C 15 was tested resulted in the development of tetanus on the third day after inoculation.

(d) A control mouse, inoculated with the *Welchii* toxin alone, remained well.

The experiment was repeated using a smaller dose of *Welchii* toxin, and substantially the same result was obtained.

Commenting on these results, it is remarkable that there is such definite evidence of loss of toxogenic power, when the Type I culture is grown in a mixture along with the cultures under consideration. It is also remarkable that a similar result was not obtained with the Type II and Type III bacilli—Experiment (iii), p. 146.

This finding might suggest that cultures of Type I bacilli were more susceptible to depression of their toxogenicity by certain mixtures than were cultures of Type II and III. This is possibly so; but, short of carrying out a long series of quantitative experiments with numerous strains of each Type, such a deduction is not permissible. It is more probable that the particular strain of Type I used was—perhaps only temporarily—less toxogenic than the strains of Types II and III employed, and therefore the conditions necessary for demonstrating depression of their toxogenicity were not realised.

The important point is, as the experiments definitely show, that certain mixtures of organisms depress to a greater or less extent the toxin-producing capacity of certain strains of tetanus bacilli when grown together with them. Experiment (vi), p. 142, of the series shows that spores of tetanus bacilli obtained from such non-toxic mixtures are definitely toxogenic when inoculated into animals under suitable conditions.

(d) PRELIMINARY EXPERIMENTS CONDUCTED WITH A VIEW TO THE  
ELABORATION OF A METHOD FOR STUDYING INFECTION  
WITH *B. TETANI*.

*Investigations conducted for the purpose of determining a suitable tissue debilitant, which might be employed for starting the infective process in various animals.*

The experiments already described indicate that a method might be elaborated for studying the various problems of the prophylaxis and therapeutics of tetanus from the standpoint of *infection* instead of from the standpoint of *intoxication*.

Could this be done, a more rational view of the pathogeny of the natural disease would be obtained, and, it is not improbable, that improvements in the methods of prevention, if not in the methods of treatment, might be suggested by the findings obtained.

The primary object of the investigation was to obtain, if possible, a standard method of *infection*. This would constitute a basis for the study of the prophylactic values of *anti-bacterial* and *antitoxic* sera; and also for the study of the action of "mono-typical" sera in order to determine whether they are or are not specific.

The experiments described on p. 142 show that the toxin of *B. Welchii* might be used for this purpose in guinea-pigs and in mice; it also sets up infection in the rat. The employment, however, of a biological reagent of this nature, for the purpose in view, presents considerable difficulties, owing to its instability, and owing also to its somewhat inconstant



effect upon different members of even one animal species, because of the variation in susceptibility exhibited. I have employed it in conducting a series of observations which will be described later, but found it unsatisfactory; because, if reliable results are to be obtained, it is essential that a complete series of experiments be performed at one time. This precluded its use in experiments in which guinea-pigs were employed, owing to the expense incurred, and, as will be seen from subsequent observations, infection experiments are difficult to carry out under standard conditions.

When a standard number of spores together with the debilitant are injected into animals, it is found that a narrow margin exists between the degrees of tissue debility which, on the one hand, will certainly give rise to the development of tetanus and which, on the other hand, will cause death in most animals even though protected by large doses of antitoxin.

By the term "a large dose of antitoxin," I mean such a (relative) quantity as would be quite impracticable as a prophylactic dosage in man; *e.g.* 50 units for a guinea-pig is equal to 12,000 units for man.

A number of substances were therefore investigated in an attempt to obtain a more stable, and therefore more constant irritant than *Welchii toxin*, or any other biological product, and which could moreover be standardised by physical or chemical methods. The substances examined were:

1. *Lactic Acid*. Various solutions of this reagent were tried both on guinea-pigs and mice, which were inoculated at the same time with a standard number of spores. The results were so inconstant that the reagent was obviously unsuitable for the purpose in view.

2. *Trimethylamine*. This reagent was also tried and was found to give fairly constant results in the case of guinea-pigs; but its marked alkalinity and its tendency to undergo decomposition proved it to be a less reliable substance than saponin for the purpose of setting up infection in these animals.

Trimethylamine could not be satisfactorily used in the case of mice, for a somewhat unexpected result was obtained with them, illustrating the difficulty of the problem under consideration, and which I think is worthy of record.

Twelve mice were inoculated subcutaneously with the following mixtures of spores and trimethylamine.

(a)	100 million	Type I	spores	+ 0.1 c.c.	trimethylamine	33 %	+ -
(b)	"	"	II	" + "	"	"	+ -
(c)	"	"	III	" + "	"	"	+ -
(a')	"	"	I	" + 0.05 c.c.	"	"	+ 0.05 saline
(b')	"	"	II	" + "	"	"	+ "
(c')	"	"	III	" + "	"	"	+ "
(a'')	"	"	I	" + 0.025 c.c.	"	"	+ 0.075 saline
(b'')	"	"	II	" + "	"	"	+ "
(c'')	"	"	III	" + "	"	"	+ "
(a''')	"	"	I	" + 0.0125 c.c.	"	"	+ 0.0875 saline
(b''')	"	"	II	" + "	"	"	+ "
(c''')	"	"	III	" + "	"	"	+ "

The volume of each *inoculum* was 0.3 c.c.

(1) Animals (*a*), (*b*), and (*c*), were all found dead the morning following the inoculation. They showed no evidence of tetanus.

(2) (*a'*), (*b'*), and (*c'*), each developed an eschar about  $\frac{1}{4}$  of an inch in diameter at the site of injection, two days after inoculation. Notwithstanding this, they remained well, were lively, took food greedily, and after a further lapse of three or four days, all but one had recovered. The animal which died—(*b'*)—did not show any evidence of tetanus, and only succumbed after the eschar had completely healed.

(3) (*a''*) developed definite tetanus on the 7th day after inoculation and was killed.

(*b''*) showed demonstrable tetanic spasm on the 3rd day and it, too, was killed.

(*c''*) died from tetanus on the 2nd day.

(4) (*a'''*), (*b'''*), and (*c'''*), all remained well.

From the above experiments it is seen that only the third series of tests gives results which are at all encouraging; but the time variation before the onset of the disease, makes the employment of diluted trimethylamine impracticable for experiments designed to investigate the prophylactic value of sera.

It is very remarkable that those animals which received 0.05 c.c. of the reagent, although they showed marked local disturbance, did not succumb to tetanus; while those which received 0.025 succumbed although they showed practically no local disturbance.

3. *Saponin*. The use of this reagent was suggested to me by Dr F. Ransom and I wish to thank him cordially for his advice.

The following experiments were carried out to determine how this substance might be employed in order to set up infection. It is to be noted, that the great advantage of saponin lies in the fact that its solutions can be standardised by *physical* methods, and it can be sterilised by autoclaving, without losing its irritant properties; solutions should not, however, be autoclaved more than once.

Five guinea-pigs were inoculated intramuscularly with the following mixtures:

Animal 1,	saponin	1/100	0.2 c.c.,	saline	0.2 c.c.,	Type I spores	200 million—	Vol.	= 0.6 c.c.
" 2,	"	1/200	"	"	"	"	200	"	" "
" 3,	"	1/400	"	"	"	"	200	"	" "
" 4,	"	1/800	"	"	"	"	400	"	" "
" 5,	"	1/1600	"	"	"	"	400	"	" "

*Number 1* showed evidence of local tetanus on the morning of the 2nd day, rapidly developed tetanic convulsions and was therefore killed.

*Number 2* had stiffening of the inoculated limb on the evening of the 2nd day and was found suffering from generalised tetanus on the morning of the 3rd day. It was then killed, as the possibility of recovery was remote.

*Numbers 3, 4 and 5* remained perfectly well. This experiment gave an indication of how a standard infection might be set up in the guinea-pig—

at least so far as a standard infection of any kind can be set up in any animal. Subsequent experiment showed that 200 million spores + 0.2 c.c. of 1/200 saponin gave consistent results, tetanus invariably developing on the 2nd or 3rd day after the intramuscular injection of the mixture. Equal volumes of 1/300 dilution of the same sample of saponin in presence of the same number of spores did not give constant results.

A similar series of tests was made with mice, dilutions of saponin 1/50–1/400 being tested, but the results obtained were entirely negative. The test was done in triplicate, using spores of all three types of *B. tetani*. The animals remained well when the lower concentrations were used, and showed absolutely no evidence of tetanus, but succumbed to saponin intoxication, usually within 24 hours, when the higher concentrations were injected.

The results of these experiments, dealing with the employment of irritants for setting up infection, show the following interesting points.

(i) *Welchii* toxin, although unsatisfactory for performing quantitative tests, proved to be the most consistent of the reagents investigated, in respect of its power of producing infection in a variety of animal species.

(ii) The chemical reagents are less consistent, *e.g.* saponin sets up infection in the guinea-pig but not in the mouse.

(iii) The dilution of the reagent employed may have a very marked influence, and sometimes an unexpected one, in respect of its power to give rise to infection in different animals. This fact is strikingly illustrated in the case of trimethylamine. When this reagent is used on mice it shows, that not only the degree, but also the nature of the tissue-debilitating lesion may profoundly affect the development of tetanus.

(iv) In the guinea-pig, saponin can be used to set up an infection, and gives fairly constant results. The animals die two to three days after inoculation. A method of testing the *anti-infective* properties of sera in guinea-pigs seems, therefore, to have been obtained.

(v) The experiment, in which the infection-stimulating properties of saponin were investigated, shows that the degree of tissue destruction, rather than the number of spores inoculated, is the determining factor in causing the development of *infection*. Thus animals 4 and 5 did not develop the disease although they received twice the number of spores injected into animals 1, 2 and 3.

#### (c) EXPERIMENTS DEALING WITH PROPHYLAXIS WITH ANTI-TETANIC SERUM.

(i) *Degree of tissue destruction in its relation to the causation of tetanus.*

(ii) *Degree of immunity conferred by varying the dose of antitoxin.*

In the previous Sub-section, pp. 147–150, attention is called to the important bearing which the degree of tissue debility may have upon the

development of tetanus, when a constant number of spores are inoculated. This raises the question: "Does antitoxin, used in doses having a ratio practicable in man, prevent a fatal issue in experimental animals, when the degree of tissue destruction is great?"

In an attempt to inquire into this problem the following experiment was carried out:

Four rats, (a), (b), (c) and (d), each weighing approximately 100 grammes, received 40 units of antitoxin; the antitoxin used was mark "B.W. horse 13."

After an interval of two days animal

(a)	was inoculated with a mixture of <i>Welchii</i> toxin + 500 million Type I spores,
(b)	" " " + 500 " " II "
(c)	" " " + 500 " " III "
(d)	" " " + saline.

The dose of *Welchii* toxin chosen was that which produced a marked oedema of the whole limb when injected by the intramuscular route. Controls which had received no antitoxin were also injected with the same mixtures. The control animals and also animals (a), (b) and (c) all developed tetanus and died, the fatal issue in the experimental animals as compared with the control animals being delayed only for 24 to 48 hours. Animals injected with tetanus spores only remained well. Animal (d)—*Welchii* toxin alone—naturally developed a marked oedema of the inoculated limb, but recovered completely in four or five days.

The experiment was repeated on mice, which were given 5 units of antitoxin two days before the inoculation of spores and *Welchii* toxin. An exactly similar result was obtained.

If the dose of antitoxin used in these experiments be expressed as gramme weight equivalents for man the ratio in each instance is:

40 units to a rat of 100 grammes is equal to
24,000 " " man of 60 kilos.
5 " " mouse of 15 grammes is equal to
20,000 " " man of 60 kilos.

The results indicate that the development of infection with *B. tetani*, in passively immunised animals, depends largely upon *quantitative* factors, one of which is, the degree of tissue destruction in the infected area. While, therefore, we may improve upon the methods of serum prophylaxis at present in use, we cannot hope to eliminate the disease completely by the prophylactic use of antitoxin.

Another most important point is suggested by the experiment, viz. that a factor of special import in the prophylaxis of the disease, is early and free removal of devitalised tissue. *Surgery is as important as serum prophylaxis in the prevention of tetanus.*

This finding prompted a further query: "Will a large dose of antitoxin give more adequate protection than a smaller one against a fatal result from infection with *B. tetani* under standard conditions—so far as any infection can be standardised?"

This subject is of great importance and can only be investigated by experiments based on *infection*, as opposed to experiments based on *intoxication*. The point at issue is not how long the passive immunity conferred by a dose of antitoxin lasts, but the degree of immunity conferred by antitoxin during the period between the 3rd and 6th day after the reception of the injury.

It has been my experience in conducting infection experiments in guinea-pigs, that if the animal lives for six days after it has been inoculated without showing evidence of tetanus, the disease will probably not develop at all; or, if it does, the infection, in a considerable number of instances, will only result in local tetanus and subsequent recovery. Stated in terms of the natural disease in man, the question really becomes—*Can we hope to prevent the occurrence of a percentage of fulminating cases of the disease by the routine employment of a larger initial prophylactic dose of antitoxin than that which is at present in use?*

In the earlier series of infection experiments in which guinea-pigs were used, the serum was injected two or three days prior to the inoculation of the infecting material—mixture of spores and irritant—and the following results were obtained:

Table XVII.

No. of animals	Ratio of anti-toxin for use	Units	Recovered	Died
21	1,450	6 or less	0	21
2	2,900	12 units	0	2
6	6,000	25 „	1	5
6	12,000	50 „	2	4

In order further to investigate this problem I performed the following experiment:

Two sets of 3 guinea-pigs each, were immunised with various doses of antitoxin; Lister Institute, No. 136 A serum was used. Two hours before the serum was injected, all the animals were inoculated intramuscularly with a mixture of 0.2 c.c. 1/200 saponin solution + 200 million spores, the total volume of the *inoculum* being 0.6 c.c. Table XVIII, p. 153, shows the details and results of the experiment.

Commenting on these results it is seen, that with the five units of antitoxin the onset of the disease was not delayed so markedly as when larger quantities were employed. With 10 units of antitoxin the late onset of the disease and its relatively slow progress indicate that a larger initial prophylactic dose of antitoxin will probably be of considerable value, both in limiting the incidence of the disease and in beneficially altering its clinical characters, by rendering the latter more chronic and so allowing of more adequate therapeutics.

Table XVIII.

Animal	Saponin	Spores	Anti-toxin	Ratio	Result
A	1/200 0.2 c.c.	200 million Type I	5	1200	Local tetanus 2nd day, generalised 4th day
B	"	" " I	10	2400	Remained well till 13th day, developed local tetanus—generalised three days later
C	"	" " I	20	4800	do. do.
A'	"	" " II	5	1200	Local tetanus 3rd day, definitely generalised 6th day
B'	"	" " II	10	2400	Remained well till 13th day, developed local tetanus—generalised three days later
C'	"	" " II	20	4800	Died 4th day. No evidence of tetanus

The experiments are too few in number, and the conditions under which they were carried out too variable—I refer, particularly, to the experiments synopsised in Table XVII, p. 152—to permit of any far-reaching conclusions being drawn from the results obtained; they are, nevertheless, extremely suggestive.

I would here call attention to an apparent discrepancy between the protective values of the sera as shown in the results indicated in Table XVII, p. 152 and Table XVIII, p. 153.

In the experiments shown in Table XVIII the serum appears to exert more marked protective influence than in the experiment shown in Table XVII. This may depend upon the fact, that the serum and the infecting material were inoculated on different days in the first instance, and on the same day in the second instance.

The obvious criticism which might be made of these experiments is, that the ratios of antitoxin used are so high, that the figures are of no import. It must be clearly appreciated, however, that the conditions which determine infection with *B. tetani* in the guinea-pig, are almost certainly very different from those which determine infection in man. One of the most striking features of the infection experiments which I have performed, is the marked degree of tissue debility that must be induced to ensure infection in these animals. It is well known that in unprotected men, on the contrary, a comparatively small lesion may suffice to set up a fatal infection with *B. tetani*.

Further, it is very questionable what interpretations we should put upon the ratios expressed in these Tables, for clearly, a dose of 500 units, for example, will only be neutralised by ten times the quantity of toxin required to neutralise a dose of 50 units. But man is certainly more susceptible to tetanus toxin (spasmin)—probably *much* more susceptible—than the guinea-pig; and there is some ground for believing that he is also much more susceptible to infection. Therefore the ratios stated in Tables XVII and XVIII may not be so fantastic as they appear to be.

Moreover, the extent of the exposed surfaces in large wounds in men, probably tends to make a gramme weight basis of comparison between man and guinea-pig a more correct index of the relative conditions obtaining in the two species, than would a simple "dose for dose" comparison.

It is these factors which make protection experiments in guinea-pigs, when tested by the infection method, extremely difficult to interpret. For with the excessive tissue destruction which is required to ensure infection in that animal, the tetanus bacilli develop in a situation essentially *outside* the body. By reason of their development in this situation, they are protected from the body-fluids, and probably grow with extreme rapidity. In a certain number of cases of the natural disease as it occurs in man, when the tissue lesion is less extensive, the contact between the living tissue and the organism may be fairly intimate. In such circumstances reaction to infection may play a not unimportant part in preventing the occurrence of the disease.

Another point which must not be lost sight of, is, that the concentration of spores in the circumscribed, though relatively large, devitalised area, in experimental infection, is much greater than is likely to occur in most cases of natural infection. This will naturally tend to make the experimental disease exhibit a fulminating character; but unfortunately, a large number of spores—200 million were used throughout the series of tests—appears to be necessary to ensure infection. With small numbers one cannot be certain of introducing a sufficiency of viable spores, to cause death of control unprotected animals within three days from inoculation.

The setting of a time limit of three days for the controls is, of course, arbitrary; but, unless a short period be taken for this purpose, the experimental error assumes serious proportions, owing to the number of survivals which occur, even in the control animals, when the period between inoculation and onset of the disease is lengthened.

#### CONCLUSIONS.

1. As one would naturally expect, the extent and degree of tissue devitalisation are an extremely important factor in determining the occurrence and termination of infection with *B. tetani*.

2. If the extent and degree of devitalisation exceed certain limits, it is certain that in animals—therefore also probably in man—no amount of antitoxin within practical limits, will give complete protection from the disease.

3. There is some evidence that the administration of a large initial prophylactic dose of antitoxin may give adequate protection against tetanus to a larger number of men than a small dose. What I mean is, that while a prophylactic dose of, for example, 500 units, will prevent the occurrence of a certain high percentage of tetanus cases among men, an increased dose to 1000 units would not result in the prevention of double the number of cases.

It would, however, probably increase the percentage of successful applications of serum prophylaxis, although the increase in percentage may be only small.

(f) INVESTIGATION OF ANTI-INFECTIVE PROPERTIES OF ANTITOXIC AND ANTI-BACTERIAL SERA.

Experiments undertaken to determine

(i) *Whether antitoxic sera exhibit anti-infective qualities in relation to the serological Type of the infecting bacilli.*

(ii) *Whether improvement in serum prophylaxis may be looked for from the employment of sera possessing anti-bacterial as well as anti-toxic properties.*

The experiments carried out *in vitro* which were discussed in the previous Section of the Report, pp. 120-135, show that *antitoxic* sera neither exhibit specific neutralising properties to the spasm-producing toxins of the three Types, nor do they stimulate phagocytosis of *B. tetani*. The experiments did suggest however, that antitoxic serum might, on the contrary, specifically neutralise the leucotoxic quality possessed by certain specimens of toxin.

It seemed possible, though improbable, that *mono-typical antitoxic* sera might, under certain circumstances, exhibit *anti-infective* properties specific to the Type of the infecting organism.

Experiments were undertaken with a view to investigating this point.

A. INFECTION EXPERIMENTS IN WHICH *WELCHII* TOXIN WAS USED AS THE TISSUE DEBILITANT.

*Experiment VIII.*

Six guinea-pigs, 1, 2, 3, 4, 5 and 6, were used; animals 1, 2 and 3 each received 2 c.c. normal rabbit serum while 4, 5 and 6 were passively immunised with 2 c.c.—50 units—of the serum of a rabbit prepared with Type II toxin.

On the second day after the administration of the serum, 200 millions of spores were injected by the intramuscular route, together with *Welchii* toxin. The dose of *Welchii* toxin in each instance was 0.1 c.c. = 1/2 the mouse M.L.D. of the particular toxin employed. The details of the experiment and the results obtained are shown in the following Table.

Animal No.	Spores 200 million, <i>Welchii</i> toxin 0.1 c.c., volume = 0.6 c.c.	Serum used, volume 2 c.c., antitoxin 50 units	Result
1	Type I	Normal rabbit	Died from tetanus 2nd day after inoculation
2	„ II	„	do. do.
3	„ III	„	do. do.
4	„ I	Type II antitoxin	Generalised tetanus 3rd day
5	„ II	„ „	Remained well
6	„ III	„ „	Generalised tetanus 3rd day



*Experiment IX.*

The result of Experiment VIII was encouraging, and it was therefore decided to repeat the test with mice. In this instance, the *antitoxic* sera were mixed with the spores and the *Welchii* toxin, and all three reagents were injected together. The following are the details of the experiment.

*Inocula* (a) spores = 100 million.

„ (b) antitoxin = 0.6 units.

„ (c) *Welchii* toxin = 1/2 mouse M.L.D.

Antitoxin Type I = Horse 13 B.W.

„ Type II = Rabbit I'.

Inoculation of spores, antitoxin, and tissue debilitant, made together.

Animal No.	Spores Type	Antitoxin Type	<i>Welchii</i> toxin	Result
1	I	Nil—saline only	1/2 M.L.D.	Dead 2nd day
2	II	„ „	„	„
3	III	„ „	„	„
4	I	Type I—0.6 units	„	Remained well
5	II	„ „	„	Generalised tetanus 4th day
6	III	„ „	„	„ „ 5th „
7	I	Type II—0.6 units	„	General tetanus 2nd day
8	II	„ „	„	Remained well
9	III	„ „	„	Generalised tetanus 3rd day
10	Nil	Nil	„	Ill for one day but recovered

*Note.* In the above experiment, the Type I antitoxin was obtained from Dr O'Brien and the vehicle of the antitoxin was therefore horse serum. It is not strictly comparable therefore with the Type II antitoxin which was prepared by myself—the vehicle of the antitoxin being rabbit serum. I here wish to record my thanks to Dr O'Brien for placing this serum at my disposal, as my Type I rabbits were not available for use at that time.

The above results are very striking, but the experiments might justifiably be criticised on the following grounds:

1. It may be purely a coincidence that the *homologous* animals survived. Admittedly the chance of such occurring is remote; but, in view of the complexity of the experiments, it cannot be excluded.

2. Using the toxin of *B. Welchii*—a biological reagent—as the tissue debilitant, introduces a potential error, for it is probable that not every individual of a species is equally susceptible to this toxin. Many attempts have been made to repeat the experiment, but using chemical agents to light up the infection. None of these, however, proved successful; for, either the controls did not take the disease, or the amount of tissue debilitant that had to be introduced to ensure infection, resulted in the death of a number of the animals, not from tetanus, but from poisoning with the chemical agent employed.

3. The amount of *Welchii* toxin used—1/2 M.L.D.—appears to be excessive; but again, it is necessary if infection is to occur regularly. Such a large dose of tissue debilitant leaves but a slight margin to allow of accidents. In an attempt to overcome certain of these criticisms the experiment was repeated on guinea-pigs.

This test was performed one week after the completion of Experiments VIII and IX; the same specimen of *Welchii* toxin was employed, in the same dose, *by volume*, as before. When this experiment was carried out, I did not fully appreciate the rapidity with which *Welchii* toxin deteriorates. As the results of Experiment X show, this deterioration of toxicity really made the test invalid.

I quote the experiment to illustrate one of the many difficulties which are encountered in conducting infection experiments with *B. tetani*, and to emphasise how very difficult it may be to interpret the results obtained.

#### *Experiment X.*

The details of this experiment are shown as follows.

- (a) Animals = guinea-pigs.
- (b) Spores dose = 250 million.
- (c) *Welchii* toxin = same dose, and same volume of toxin as used in Experiment VIII.
- (d) Antitoxin Type I = Horse 13 B.W.
- (e)       "       "   II = Rabbit I'.
- (f) Dose of antitoxin = 5 units.
- (g) Method of inoculation -- Mixtures made of spores, *Welchii* toxin and tetanus antitoxin, incubated for 30 minutes at 37° C. and injected simultaneously by the intramuscular route.

Animal No.	Spores Type	<i>Welchii</i> toxin	Antitoxin 5 units	Result
1	I	"	Nil—saline only	Local tetanus 9th day, generalised 14th day
2	II	"	" "	General tetanus 8th day
3	III	"	" "	do. do.
4	I	"	Type I antitoxin	General tetanus 10th day
5	II	"	" "	Animal remained well
6	III	"	" "	General tetanus 10th day
7	I	"	Type II antitoxin	General tetanus 10th day
8	II	"	" "	Animal remained well
9	III	"	" "	General tetanus 9th day

This experiment is really valueless, for the time which elapsed between making the inoculation and the onset of the disease in the control animals Nos. 1, 2, and 3, was so long, that the passive immunity in the experimental animals must have almost disappeared.

I would call attention to the fact, that both animals 5 and 8 remained well. This suggested that Experiment VIII should be accepted with a certain reserve, as it appeared possible, that the infectivity of the Type II spores used might be somewhat less than that of the Type I and Type III spores. In view of the result of Experiment IX, I do not think that this was the

case in Experiment VIII, but the possibility of such an interpretation of the results at any stage of the investigation, must be borne in mind.

The experiment was then repeated, and on this occasion sufficient *Welchii* toxin was administered to cause swelling of the whole of the inoculated limb. The result was that none of the animals survived, and the protected animals took the disease almost as soon as the control animals. These tests on guinea-pigs and three other tests which were carried out on mice, convinced me, that one could not hope to obtain consistent results if *Welchii* toxin were employed as the tissue debilitating factor in the mixtures, owing to the difficulty of regulating the dose.

*Note.* Experiments VIII, IX, and X, and their repetitions were carried out with sample bleedings—two different samples were used—from Rabbit I', while the animal was still undergoing immunisation. Before the next series of tests was made the animal had been bled out.

It was at this stage of the investigation that I decided to change my technique, and in guinea-pigs at least, to employ chemical reagents as the tissue debilitating factor of the infecting mixtures.

Several chemical agents were tried, but saponin gave the most constant results, and was fairly easily handled, so that this substance was chosen for future investigations.

## B. EXPERIMENTS IN WHICH SAPONIN WAS USED AS THE TISSUE DEBILITANT AND THE ANTI-INFECTIVE QUALITY OF ANTITOXIN WAS UNDER INVESTIGATION.

### *Experiment XI.*

In this experiment the animals were passively immunised with 25 units of antitoxin, and two days later, were inoculated by the intramuscular route with a mixture of saponin and spores.

- (a) Animals = guinea-pigs.
- (b) Type I antitoxin = B.W. Horse 13.
- (c) " II " = Rabbit I'.
- (d) Antitoxin dose = 25 units.
- (e) " administered 48 hours before inoculations were made.
- (f) Spores = 200 million.
- (g) Saponin = 0.2 c.c. of 1/200 dilution.
- (h) Volume of infecting *inoculum* = 0.6 c.c.

Animal No.	Spores Type	Saponin	Antitoxin units 25	Result
1	I	0.2 c.c. 1/200	Nil—saline only	Generalised tetanus 2nd day
2	II	"	" "	" " "
3	III	"	" "	" " "
4	I	"	Type I	Generalised tetanus 11th day
5	II	"	"	Generalised tetanus 3rd day
6	III	"	"	" " "
7	I	"	Type II	" " "
8	II	"	"	" " "
9	III	"	"	" " "

The experiment was then performed by the method of making simultaneous inoculation of serum, debilitant and spores. The details are as follows.

*Experiment XII.*

- (a) Animals = guinea-pigs.
- (b) Spores = 200 million.
- (c) Saponin = 0.1 c.c. of 1/100.
- (d) Antitoxin = 5 units.
- (e) Total volume of *inoculum* = 0.6 c.c.
- (f) Inoculation of antitoxin and infecting mixture made simultaneously.
- (g) Antitoxic sera employed were the same as in Experiment XI.

Animal No.	Spores Type	Saponin	Antitoxin 5 units	Result
1	I	0.1 c.c. of 1/100	Nil - saline only	Generalised tetanus 2nd day
2	II	" "	" "	" " "
3	III	" "	" "	" " "
4	I	" "	Type I	Animal developed local tetanus 14th, generalised 15th day
5	II	" "	" "	Generalised tetanus 3rd day
6	III	" "	" "	" " "
7	I	" "	Type II	Generalised tetanus 2nd day
8	II	" "	" "	" " 4th "
9	III	" "	" "	" " 2nd "

That portion of the experiment dealing with Type II antitoxin was repeated twice as the above result did not corroborate Experiments VIII and IX in which *Welchii* toxin had been used as the tissue debilitant. Both experiments failed to show definite protection against infection; either *general*, in relation to all the Types, or specific, in respect of the *homologous* Type.

Examination of this serum showed that it was contaminated with a diplococcus. The serum was filtered, and the experiments were discontinued until such time as complete crossed experiments could be performed, using both *anti-bacterial* and *antitoxic* sera.

In these experiments the following points call for comment:

1. I failed to corroborate the findings of Experiment VIII in respect of Type II serum, both when the serum was administered before inoculation of the infecting mixture and when it was administered simultaneously therewith.

2. It is significant, on the other hand, that Type I antitoxin did protect for a much longer period against infection with Type I spores, than did Type II antitoxin.

3. It is remarkable that a dose of 5 units of antitoxin, when administered simultaneously with the infecting mixture, appears to give almost as much protection against infection, as does a dose of 25 units of the same antitoxin administered two days prior to the inoculation of the infecting mixture.

Before leaving this subject I decided to repeat Experiment VIII, using Type I antitoxin, and saponin as the irritant. My reason for so doing was, that in neither Experiment XI nor XII did I succeed in obtaining an unequivocal result. In performing this further experiment, the test animals

were passively immunised with 50 units of antitoxin and were inoculated with the saponin-spore mixture two days later.

*Experiment XIII.*

- (a) Animals = guinea-pigs.
- (b) Spores = 200 million.
- (c) Saponin = 0.1 c.c. of 1/100 dilution.
- (d) Volume of *inoculum* = 0.6 c.c.
- (e) Antitoxin used = B.W. Horse 13.
- (f) Dose of antitoxin = 50 units.
- (g) Antitoxin administered two days before infecting inoculations were made.

Animal No.	Spores Type	Saponin	Antitoxin 50 units	Result
1	I	0.1 c.c. of 1/100	Nil—saline only	General tetanus 2nd day
2	II	"	" "	" " "
3	III	"	" "	" " "
4	I	"	Type I B.W. 13	Remained well till 12th day, died 13th, streptococci recovered from local lesions and internal organs
5	II	"	"	Local tetanus 4th day, generalised 8th day
6	III	"	"	Local tetanus 4th day, generalised 5th day

The following deductions seem permissible from experiments VIII-XIII, pp. 155-160:

1. That *antitoxic* serum may under certain conditions exhibit *anti-infective* properties; and

2. While the experiments afford no *proof* that a monotypical antitoxic serum protects more adequately against *infection* due to serologically *homologous* strains of the bacillus than against that due to *heterologous* strains, the results obtained nevertheless *suggest* that protection against the *homologue* is more adequate.

These deductions are made in a spirit of modesty, for one is well aware that, in infection experiments, and especially with the anaërobcs, even the known factors upon which the process depends are very complex. Short of obtaining a 100 per cent. result in a considerable series of experiments, it is rash to conclude that proof of any thesis has been established.

*Does serum possessing anti-bacterial properties exhibit more adequate prophylactic qualities than serum which possesses only antitoxic (anti-spasmin) properties?*

*Note.* While the term "unit" as previously employed refers to the U.S.A. Unit, I wish especially to call attention to the fact that, in the experiments of the following Sub-section, the term "unit" is defined as "that quantity of antitoxin which will protect a mouse against 1000 M.L.D. of tetanus toxin."

The investigation of this problem involved an inquiry into the following points:

(i) Does serum possessing demonstrable *anti-bacterial* qualities (e.g. agglutinin and phagocyte stimulating properties) but of no, or only negligible antitoxic value, exhibit any capacity for preventing *infection*?

(ii) We know that *antitoxin* has a certain *anti-infective* power. Can this be enhanced by adding serum containing *anti-bacterial* immune bodies? If antitoxic (anti-spasmin) content be made the basis of assessing the probable prophylactic value of sera, does a serum of  $x$  "anti-spasmin" units which has no *anti-bacterial* qualities, protect against infection as effectively as a serum of  $x$  "anti-spasmin" units, which does possess *anti-bacterial* properties?

(iii) Can specific protection be demonstrated *in vivo* with such *anti-bacterial* sera?

The consideration of these points is of extreme import; for, should any of these questions be answered in the affirmative, the method of standardising prophylactic sera will demand review. It is only rational that, if a satisfactory method could be devised for examining sera designed for prophylactic use, these sera should be standardised on the basis of prevention of *infection*, rather than of *intoxication*.

The first experiments made were designed to corroborate the findings already obtained, and to determine a method of procedure which could be satisfactorily used for the purpose in view.

#### Experiment XIV.

This experiment was carried out as a preliminary to more careful and exact tests, in which guinea-pigs were to be used. In this instance mice were the experimental animals employed. The tissue irritant chosen was therefore *Welchii* toxin and a complete series of crossed tests was made, both with monotypical *antitoxic* and monotypical *anti-bacterial* sera corresponding to three serological Types of *B. tetani*.

The details of the experiments are as follows:

- (a) Animals = mice.
- (b) Tissue irritant = *Welchii* toxin  $< 1/2$  but  $> 1/4$  M.L.D.
- (c) Spores = 100 million.
- (d) Inoculation = *Sub cutem*.
- (e) *Antitoxic* sera dose = 1 "unit."
- (f) *Anti-bacterial* sera dose = antitoxic content of  $< 1$  "unit" and  $> 1/2$  "unit."
- (g) Sera administered one day prior to inoculation of the infecting mixtures.
- (h) Volume of infecting *inoculum* = 0.2 c.c.

#### Type I Spores.

1.	<i>Welchii</i> toxin nil	Serum nil	Died 5th day, no evidence of tetanus
2.	<i>Welchii</i> toxin	+ serum nil	Remained well
3.	" "	+ Type I <i>antitoxin</i>	" "
4.	" "	+ " II "	Died 2nd day—? tetanus
5.	" "	+ " III "	" " "
6.	" "	+ " I <i>anti-bacterial</i>	Remained well
7.	" "	+ " II "	" "
8.	" "	+ " III "	Died 2nd day—? tetanus

## Type II Spores.

1. <i>Welchii</i> toxin nil	Serum nil	Remained well
2. <i>Welchii</i> toxin	"	Definite tetanus 3rd day, died
3. " "	+ Type I antitoxic serum	Definite tetanus 4th day
4. " "	+ " II " "	Remained well
5. " "	+ " III " "	" "
6. " "	+ " I anti-bacterial serum	" "
7. " "	+ " II " "	" "
8. " "	+ " III " "	Definite tetanus 3rd day

## Type III Spores.

1. Nil	Nil	Died 4th day, no evidence of tetanus
2. <i>Welchii</i> toxin	"	Definite tetanus 3rd day
3. " "	+ Type I antitoxic serum	" "
4. " "	+ " II " "	" "
5. " "	+ " III " "	Remained well
6. " "	+ " I anti-bacterial serum	Died 3rd day, ? evidence of tetanus
7. " "	+ " II " "	Remained well
8. " "	+ " III " "	" "

The results of Experiment XIV may be summarised thus:

(i) Of those animals which received spores *homologous* to antitoxic sera employed, all three remained well.

(ii) Of six which received spores *heterologous* to the antitoxin three developed definite tetanus and died; two others died but evidence of death from tetanus was lacking, and one remained well.

(iii) Of nine animals which received *anti-bacterial* sera, one showed definite tetanus; two died but evidence of tetanus was lacking, and six remained well.

Considering that the unitage of the *anti-bacterial* sera in the above experiment was certainly less than that of the *antitoxic* sera, it is notable that so many survivals occurred among the animals protected with the former.

The section of the experiment which deals with Type I spores is, however, really valueless, in that infection did not "take" in the control.

If then we consider only those sections of this experiment which deal with Type II and Type III spores, it is seen that

(a) Of six mice protected with *antitoxic* sera, three developed tetanus and three remained well.

Two of the surviving mice were protected by sera *homologous* to the infecting organisms; in one the infection was *heterologous*.

(b) Of six animals protected by *anti-bacterial* sera, only one developed tetanus and four remained well. One mouse died on the third day after inoculation but evidence of death from tetanus was lacking. Both of those in which the sera and the infecting organisms were *homologous* were included among the four mice which remained well.

Here again is a suggestion, but no proof, that *monotypical* antitoxic sera may, under certain circumstances, exhibit a specific influence on infection from *serologically homologous* bacilli.

(c) There is also an indication that *anti-bacterial* sera exhibit more adequate *anti-infective* properties than do *antitoxic* sera, provided that the "anti-spasmin" value of the sera be made the basis of comparison.

*Note.* There is some ground for believing that mice are not very susceptible to tetanus *infection*. When these animals are employed, the assessment of the results obtained is rendered especially difficult.

Far-reaching conclusions cannot be drawn from the results obtained.

The above experiment, which lays no claim to strict accuracy, was valuable however, in that it served to indicate how to proceed further and showed that the thesis advanced was worthy of extended investigation.

Henceforth guinea-pigs only were employed as the experimental animals, and the irritant used was saponin. More reliable results could be expected with this technique, than when mice and *Welchii* toxin were used.

### Experiment XV.

This experiment was carried out to determine whether *anti-bacterial* sera, possessing little or no antitoxic properties, would protect against *infection*. Sample bleedings were made from Rabbits III, III' and III'', "anti-whole culture" serum to Types I, II and III respectively. These sera, retained from a previous investigation, showed some phagocytic properties when diluted 1/200, and their antitoxic value was equal to about 2 "units" per c.c. With these serum samples the following experiment was carried out.

- (a) Animals = guinea-pigs.
- (b) Spores = 200 million.
- (c) Tissue irritant = 0.1 c.c. of 1/100 saponin.
- (d) Inoculation = intramuscular.
- (e) *Anti-bacterial* sera Type I, Type II and Type III (equivalent of 1 "anti-spasmin" "unit" used).
- (f) *Antitoxic* serum = Type I (equivalent of 6 "anti-spasmin" "units" used).
- (g) Sera administered one day prior to inoculation of infecting mixture.
- (h) Volume of infecting *inoculum* = 0.6 c.c.

Animal No.	Spores	Serum	Result
1	I	Nil	General tetanus 4th day
2	II	"	" " 3rd "
3	III	"	Animal remained well
4	I	6 "units" Type I <i>antitoxic</i>	General tetanus 7th day
5	II	" " "	" " 3rd "
6	III	" " "	" " 7th "
7	I	1 "unit" Type I <i>anti-bacterial</i>	" " 3rd "
8	II	" " "	" " 3rd "
9	III	" " "	" " 3rd "
10	I	1 "unit" Type II <i>anti-bacterial</i>	" " 4th "
11	II	" " "	" " 2nd "
12	III	" " "	" " 3rd "
13	I	1 "unit" Type III <i>anti-bacterial</i>	" " 3rd "
14	II	" " "	" " 2nd "
15	III	" " "	" " 3rd "



Experiment XV shows that sera which possess *anti-bacterial* properties will not serve to prevent a fatal issue in an experimental infection set up by the method described, if the sera do not also possess sufficient *antitoxic* qualities.

This fact was confirmed by investigations made with agglutinating sera which possessed no, or negligible *antitoxic* qualities. These also failed to prevent infection under the conditions of the experiment.

I wish to call attention to the survival of the control animal which received Type III spores. In view of such survivals, extreme care must be exercised in drawing conclusions from the results of these tests.

Experiment XV was repeated with a modified technique; serum and infective material being injected simultaneously, and only *anti-bacterial* sera used. The quantity of each serum used contained 1 "unit" of antitoxin.

The result of this experiment showed, that even under these conditions, the *anti-bacterial* sera did not exert demonstrable *anti-infective* qualities in the experimental *infection* set up by the method described.

This result was somewhat unexpected; but, when it is borne in mind that saponin has an extremely deleterious influence on all the tissues, and is very markedly haemotoxic, this can be readily understood. For, during the first day or two after inoculation, *B. tetani* develops virtually outside the body, and may elaborate enough toxin to kill the animal before sufficient local reaction is set up in the tissues to deal with the process of infection. If it were possible to combat this initial intoxication, and at the same time favourably influence the tissues to deal with the local infective process, better results might be obtained.

With this object in view the following experiment was undertaken.

### Experiment XVI.

Four guinea-pigs, A, B, C and D, each received 20 "units" of Type III antitoxin (*i.e.* antitoxin prepared by inoculation of the toxin of Type III bacilli).

The following day

A was inoculated with a mixture of saponin and Type I spores.

B	"	"	"	"	I	"
C	"	"	"	"	III	"
D	"	"	"	"	III	"

Two days were allowed to elapse and then,

A was given a dose of 2 "units" of Type III *anti-bacterial* serum.

B	"	"	2	"	"	<i>antitoxic</i>	"
C	"	"	2	"	"	<i>anti-bacterial</i>	"
D	"	"	2	"	"	<i>antitoxic</i>	"

Animals A, C and D remained well, while B developed generalised tetanus

six days after the inoculation of the infective mixture. In this experiment then,

- (a) The animal which received *homologous* antitoxin survived.
- (b) The animal which received only *heterologous* antitoxin died from tetanus.
- (c) Both animals, which on the second day after inoculation of infective material received a dose of *anti-bacterial* serum, recovered.

In this experiment too large an initial dose of antitoxin was used to permit of its being satisfactorily demonstrated that *anti-bacterial* sera possess more adequate *anti-infective* properties than do *antitoxic* sera.

In Experiment XVII an effort was made to overcome this difficulty by using a smaller dose of serum.

#### *Experiment XVII.*

Four guinea-pigs A, B, C and D were passively immunised as follows:

A received 12 "units" of Type III *antitoxic* serum

B    "      6    "       "       "       + 4 "units" of Type III *anti-bacterial* serum

C    "      12   "       "       *antitoxic* serum

D    "      6    "       "       "       + 4 "units" of *anti-bacterial* serum

The following day A and B were each inoculated intramuscularly with a mixture of saponin 0.1 c.c. of 1/100 and Type I spores 200 million, total volume 0.6 c.c. C and D each received a similar inoculation, but with Type III instead of Type I spores.

Animal A developed local tetanus on the third day after inoculation, the disease being generalised on the fifth day.

„ B developed local tetanus on the fifth day which generalised on the sixth day.

„ C developed local tetanus on the third day, general tetanus supervening on the following day.

„ D remained well.

This experiment definitely showed that *anti-bacterial* sera were worthy of extended investigation.

I would call attention to the fact, that Experiments XVI and XVII illustrate a further difficulty, which has so far not been overcome, viz. that the bacilli, from a given number of spores, are not always equally toxogenic. In Experiment XVI, Animal B (inoculated with Type I spores), although it received in all 22 "units" of antitoxin, developed generalised tetanus six days after inoculation. Whereas in Experiment XVII when the same spores, but only half the quantity of the same antitoxin, were used, generalised tetanus developed only one day earlier—the fifth day—preceded by local tetanus for a period of two days. In Experiment XVII then, the Type I spores did not appear to be so toxogenic as in Experiment XVI.

In order, finally, to examine this subject, a complete series of experiments was carried out. Great care was taken to standardise the reagents as accurately as the equipment of my laboratory would permit, and to carry out the experiments under standard conditions as far as possible.

The following are the protocols of experiments carried out with the specimen

bleedings of *antitoxic* and *anti-bacterial* sera. The sera were standardised with fair accuracy before the tests were performed.

As it was not possible to carry out the entire experiment with all six sera—three *antitoxic* and three *anti-bacterial*—on the same day, the experiment had to be divided into three sections. Each section deals with the investigation of the *anti-infective* value of all the sera in respect of only one Type of spores.

#### METHODS USED.

(1) The basis of comparison for the sera was their *antitoxic* value. This was standardised as accurately as possible; but no doubt slight differences existed between the real and the estimated unitage of each. I was unable to obtain any standard U.S.A. toxin with which to perform the preliminary tests and therefore had to adhere to the "mouse M.L.D." method of standardisation. Such differences, however, do not really affect the results, as the same ratio obtains in each experiment of the series.

(2) The quantity of antitoxin provisionally decided upon was 10 units.

(3) The sera were injected *sub-cutem* into the right hind limb.

(4) One hour after the administration of the sera, a mixture consisting of 0.1 c.c. 1/100 dilution of saponin + 200 million spores suspended in 0.6 c.c. of saline, was injected intramuscularly into the left hind limb.

(5) The animals employed were guinea-pigs.

(6) The volume of serum administered in each instance was made up to 2 c.c. by the addition of normal rabbit serum. (The *antitoxic* and *anti-bacterial* sera used in these experiments were obtained from later bleedings from the same rabbits as were used in the experiments described in Section IV.)

(7) The site of the inoculation where the saponin spore mixture was injected was dried with alcohol and sealed with collodion. This precaution is essential, because of the danger of secondary infection from the animal cage, bedding, etc. This danger is enhanced by the tissue devitalisation produced by the saponin.

#### Experiment XVIII.

(a) Type I spores used.

(b) Antitoxic titre of each serum tested—10 "units."

Number	Serum	Result
1	Normal rabbit serum 2 c.c.	Died from tetanus 3 days after inoculation
2	Type I <i>antitoxic</i> serum 10 "units"	Local tetanus developed 3rd day; persisted for 8 days. Thereafter the animal slowly recovered
3	Type II <i>antitoxic</i> serum 10 "units"	Local tetanus developed 3rd day; persisted for over three weeks
4	Type III <i>antitoxic</i> serum 10 "units"	Local tetanus 3rd day; generalised on 4th; animal killed
5	Type I <i>anti-bacterial</i> serum 10 "units"	Animal remained well
6	Type II <i>anti-bacterial</i> serum 10 "units"	Local tetanus 3rd day; generalised 4th; animal killed
7	Type III <i>anti-bacterial</i> serum 10 "units"	Local tetanus 4th day; generalised 5th; animal killed

*Experiment XIX.*

(a) Type II spores used.

(b) Antitoxic content of each serum—7.5 “units.” (In view of the survival of animals 2 and 3 in Experiment XVIII the smaller dose—7.5 “units of” antitoxin—was decided upon.)

Number	Serum	Result
1	Normal rabbit serum 2 c.c.	Died from tetanus 3 days after inoculation
2	Type I <i>antitoxin</i> 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed
3	Type II <i>antitoxin</i> 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed
4	Type III <i>antitoxin</i> 7.5 “units”	Local tetanus 3rd day; both hind legs tetanised from 5th–12th day; animal commenced to use both hind limbs 17th day; recovered
5	Type I <i>anti-bacterial</i> serum 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed
6	Type II <i>anti-bacterial</i> serum 7.5 “units”	Remained well
7	Type III <i>anti-bacterial</i> serum 7.5 “units”	Local tetanus 3rd day; generalised 4th; animal killed

*Experiment XX.*

(a) Type III spores used.

(b) Antitoxic content of each serum—10 “units.”

[The higher dose of antitoxin was reverted to in this instance, as previous experiments with Type III spores had indicated that these were probably markedly toxogenic.]

Number	Serum	Result
1	Normal rabbit serum 2 c.c.	Local tetanus 2nd day; generalised tetanus 3rd day; killed
2	Type I <i>antitoxin</i> 10 “units”	Local tetanus 2nd day; generalised tetanus 3rd day; killed
3	Type II <i>antitoxin</i> 10 “units”	Local tetanus 3rd day; did not generalise; remained for 10 days; thereafter animal slowly recovered
4	Type III <i>antitoxin</i> 10 “units”	Local tetanus 3rd day; did not generalise; remained for 10 days; thereafter animal slowly recovered
5	Type I <i>anti-bacterial</i> serum 10 “units”	Local tetanus 3rd day; did not generalise; remained for 8 days; thereafter slowly recovered
6	Type II <i>anti-bacterial</i> serum 10 “units”	Local tetanus 3rd day; generalised 4th day; animal killed
7	Type III <i>anti-bacterial</i> serum 10 “units”	Local tetanus 3rd day; generalised 4th day; animal killed

This result fails to corroborate Experiments XVIII and XIX. Animals 3 and 5, although only immunised with *heterologous* sera, survived for a longer period than the animal which received *homologous anti-bacterial* serum.

It is to be noted that animals 3, 4 and 5 were all very small guinea-pigs; they exhibited the same markings, and appear to have been, though the evidence is not definite, from the same litter. It has been noted throughout the whole series of infection experiments that small guinea-pigs are

less susceptible and give less constant results than larger animals. This probably because it is more difficult to ensure an intramuscular injection being made when the animals are small. Attention is therefore especially called to the fact that these animals which survived in Experiments XVIII and XIX were comparable to those which died in Experiment XX. Owing to lack of animals, I was unable to obtain nine which were really comparable to one another.

A further series of experiments was therefore carried out using larger animals. The animals in each test were of approximately equal weight.

Owing to a shortage of guinea-pigs these tests were confined to an examination of three sera only, each serum being examined in triplicate, so that a range of unitage could be employed in the investigation of each.

The sera examined were:

- (a) *Antitoxic* serum = Type III.
- (b) *Anti-bacterial* serum = Type II.
- (c) *Anti-bacterial* serum = Type III.

### Experiment XXI.

The details of this experiment are as follows:

- (a) Animals = guinea-pigs of approximately 350 grams.
- (b) Spores = Type III—200 million.
- (c) *Anti-spasmin* "unitage" in each case, 9, 12 and 15.
- (d) Irritant -- Saponin, 1/100, 0.1 c.c.
- (e) Volume of *inoculum* = 0.6 c.c.
- (f) Serum administered one hour before injection of spore-saponin mixture.

In this experiment, the *anti-bacterial* value of the sera employed remained constant in each test, the equivalent of 6 "units." The balance of *antitoxin* was made up of 3, 6 and 9 U.S.A. Units of a polyvalent antitoxic horse serum marked L.I.P.M. 136 A.

The following were the results obtained

Animal No.	Serum administered	Spores	Result	
		Type III	General tetanus 2nd day	
1	6 "units" Type III <i>antitoxin</i> (rabbit), 3 units L.I.P.M. 136 A		"	"
2	6 "units" Type III <i>antitoxin</i> (rabbit), 6 units L.I.P.M. 136 A	"	"	"
3	6 "units" Type III <i>antitoxin</i> (rabbit), 9 units L.I.P.M. 136 A	"	"	"
4	6 "units" Type II (rabbit) <i>anti-bacterial</i> , 3 units L.I.P.M. 136 A	"	"	"
5	6 "units" Type II (rabbit) <i>anti-bacterial</i> , 6 units L.I.P.M. 136 A	"	"	"
6	6 "units" Type II (rabbit) <i>anti-bacterial</i> , 9 units L.I.P.M. 136 A	"	"	"
7	6 "units" Type III (rabbit) <i>anti-bacterial</i> , 3 units L.I.P.M. 136 A	"	"	"
8	6 "units" Type III (rabbit) <i>anti-bacterial</i> , 6 units L.I.P.M. 136 A	"	"	"
9	6 "units" Type III (rabbit) <i>anti-bacterial</i> , 9 units L.I.P.M. 136 A	"	Local tetanus 2nd day General tetanus 4th day	

This experiment will be commented on later; but I wish to call attention to the somewhat longer survival of animal 9 as compared with animals 3 and 6.

### Experiment XXII.

In this experiment though similar to the previous one horse serum was not added to bring up the desired *anti-spasmin* content of the *anti-bacterial* sera. Both *anti-spasmin* and *anti-bacterial* content therefore varied *pari passu* one with the other.

The details of the test are substantially the same as those of Experiment XXI but 10, 20 and 30 "units" were employed instead of 9, 12 and 15.

Animal No.	Serum administered	Spores	Result
1	Type III rabbit <i>antitoxin</i> 10 "units"	Type III	General tetanus 2nd day
2	Type III rabbit <i>antitoxin</i> 20 "units"	"	" " "
3	Type III rabbit <i>antitoxin</i> 30 "units"	"	" " 3rd day
4	Type II rabbit <i>anti-bacterial</i> 10 "units"	"	" " 2nd "
5	Type II rabbit <i>anti-bacterial</i> 20 "units"	"	" " "
6	Type II rabbit <i>anti-bacterial</i> 30 "units"	"	Local tetanus 3rd day General tetanus 4th day
7	Type III rabbit <i>anti-bacterial</i> 10 "units"	"	Local tetanus 2nd day General tetanus 3rd day
8	Type III rabbit <i>anti-bacterial</i> 20 "units"	"	Local tetanus 2nd day General tetanus 3rd day
9	Type III rabbit <i>anti-bacterial</i> 30 "units"	"	Local tetanus 3rd day General tetanus 4th day

I shall not comment on these results beyond calling attention to the fact, that it is obvious, that if specificity of reaction between serum and organism exists, it is obviously *quantitative*. Experiments XVIII, XIX, XX, XXI and XXII all show the difficulty of realising the conditions necessary for the demonstration of specificity.

### SYNOPSIS OF RESULTS OBTAINED IN SECTION V (pp. 135-169).

The results obtained in the Section of the Report at present under consideration may be summarised thus:

1. The genesis of tetanus *infection* induced by experimental methods depends very largely upon the degree and extent of the tissue debility produced by the *inoculum*.

2. The nature of the tissue debilitant employed for setting up infection is an important factor in this connection; for a debilitant which lights up infection in one animal species may fail to do so in another. Thus, when mice are inoculated with mixtures of saponin and tetanus spores, these animals only occasionally develop the disease. On the contrary, when guinea-pigs are inoculated with such mixtures they seldom survive, the onset of the disease being usually early and its character fulminant.

3. The quality of the tissue debility induced is also of paramount impor-

tance in the genesis of infection. Thus trimethylamine, even though it be used in sufficient concentration to cause the development of large eschars at the site of inoculation, when injected together with tetanus spores into mice only infrequently lights up the disease; whereas the toxin of *B. Welchii*, injected in sub-lethal dose together with the same spores, almost invariably sets up tetanus in these animals, notwithstanding the fact that no obvious lesion could be demonstrated *ante-mortem*.

4. In animals protected by *antitoxin*, a rapidly fatal tetanus infection can be induced, provided that the spore-containing *inoculum* produce a sufficient degree of tissue debility of the requisite character.

5. In the case of infections arising from Type I and Type II spores (Type III spores were not included in the test), it was shown that in guinea-pigs, by increasing the prophylactic dose of *antitoxic* serum, the onset of the disease can be delayed and its course can be rendered less acute. If the increase of prophylactic dose be sufficient, *antitoxic* serum *may* completely protect these animals against an *infection* induced by the inoculation of "saponin-spore" mixture.

6. There is some evidence that *monotypical antitoxic* sera protect more adequately against infection with bacilli which are serologically *homologous*, than against infection due to bacilli which are serologically *heterologous*.

Admittedly, the experimental evidence of this is only suggestive, and this deduction is made with great reserve.

7. The experiments on the relative prophylactic value of *antitoxic* and *anti-bacterial* sera prove that this subject is worthy of careful investigation.

8. Experiments XVIII and XIX (pp. 166, 167) in which Type I and Type II spores were used, strongly suggest that sera possessing *anti-bacterial* as well as *antitoxic* properties protect more adequately than do sera exhibiting only *antitoxic* properties. The evidence of specific protection in relation to the serological Type of the infecting organism appears also to be definite in these two experiments.

9. Experiment XX (p. 167), in which Type III spores were used, failed to corroborate this finding. The experiment is however interesting, in that it indicates that there is a considerable variation of susceptibility to tetanus infection among animals of one species.

10. Experiments XXI and XXII (pp. 168, 169) give a very slight indication that in the case of Type III also, *anti-bacterial* serum affords more adequate protection than does *antitoxic* serum. There is also a suggestion in the results obtained that the protection is specific.

#### DISCUSSION OF THE RESULTS OF EXPERIMENTS OF SECTION V.

It is difficult to comment upon the results obtained in Section V, for, although suggestive, they fall very far short of establishing the validity of the thesis advanced. I wish then formally to call attention to the fact that

any deductions which have been drawn from the results so far obtained, are to be regarded as only provisional in character.

The following criticisms are applicable to the experiments described.

(a) The number of experiments performed is too small to permit of definite information being obtained on the points at issue.

(b) The method employed for setting up infection is unsatisfactory for the following reasons.

(i) To set up tissue destruction or debility by means of saponin is a highly artificial procedure.

(ii) The disturbance, which must be produced when this reagent is the debilitant employed, is so great (if infection in the control animals is to be assured), that it is extremely difficult to protect against infection, even when relatively enormous doses of serum are used prophylactically.

This large amount of tissue destruction really means that the inoculated spores develop in a situation relatively remote from the body-fluids, and in such circumstances may be protected from immune bodies circulating in the blood or lymph. As the germinated bacilli continue to develop toxin, any antitoxin that is administered may ultimately be exhausted, and tetanus supervenes.

The method which one is forced to employ has therefore grave disadvantages, and leads, probably, to too severe a test being applied to the sera examined.

(iii) It is obvious that a standard method of infection is an unattainable ideal, for spores certainly vary in respect both of their *toxogenic* capacity and of their *infective* capacity.

It is to be noted that the two factors of *toxogenicity* and *infectivity* are not identical, for the spores from some cultures which have a relatively low toxin content may, under certain circumstances, be more highly *infective* than are spores from a culture of greater toxicity.

(iv) When all other sources of error which render comparative experiments of the kind under consideration difficult to carry out are eliminated, there remain two factors which never can be standardised.

(a) The test animals will always exhibit idiosyncrasy.

(b) The *toxogenic* capacity, and also probably the *infective* capacity, of a single strain of *B. tetani* varies from culture to culture.

A very important question arises in connection with this section of the work, viz. "Can we hope for any improvement in the serotherapeutics of declared tetanus by the use of *anti-bacterial* sera?"

While it is unfortunately probable that this question will have to be answered in the negative, experiments dealing with the problem will have to be undertaken.

These experiments cannot be carried out until larger quantities of *anti-bacterial* sera are available; but arrangements are now being made for the immunisation of horses against *whole culture*. The problem will



therefore be dealt with when sufficient serum from these animals is obtained.

Since completing the experiments described in Section V, I have received information, communicated personally, from Capt. Bullock, R.A.M.C., Imperial Cancer Research Laboratories, concerning the influence of the calcium ion in initiating anaërobic infections.

I here wish to record my very deep debt to Capt. Bullock, R.A.M.C., for the information which he has placed unreservedly at my disposal before the publication of his work.

Preliminary experiments with soluble calcium salts as the "Infection initiator" both in mice and in guinea-pigs have given encouraging results; and, in view of the slight tissue destruction which occurs when calcium salts are used for this purpose, I propose to repeat Experiments XVIII, XIX and XX using Bullock's technique.

## SECTION VI.

### INVESTIGATION OF DRESSINGS IN RELATION TO ANAËROBIC INFECTIONS OF WOUNDS.

In this section of the Report are considered the results obtained in the examination of 100 wounds during the process of healing.

The object of the inquiry was to determine, if possible, the procedure or procedures, which might be recommended as useful for preventing or diminishing infection of wounds due to anaërobic bacteria.

In view of the influence which anaërobes have one upon another, the general question of all anaërobe infection, rather than the particular question of any one such infection, was investigated. The presence of *B. tetani* was, however, made the subject of detailed inquiry, as the prevalence of the various serological Types of that organism, and its incidence in wounds, constitute a most important aspect of the bacteriology of tetanus.

Owing to its being relatively easy to differentiate morphologically, and because also of its important relationship to the etiology of tetanus, the incidence of *B. Welchii* was also noted. No effort could be made, however, to determine the incidence of other anaërobes in the material examined.

#### (a) *Methods used.*

It was felt, that in making this investigation, it would be better to examine thoroughly a relatively small number of wounds, and to examine them repeatedly, than to make a single examination of a much larger number. The progress made under the various treatments could be more definitely assessed by choosing a small number of cases, that were more or less comparable to one another; that is, in so far as comparison of wounds is at all possible. Five examinations of each wound were made at weekly intervals,

unless the patient became convalescent during the period. The examinations were conducted as follows:

- (i) Swabs were taken as soon after the arrival of a convoy as possible.
- (ii) Thereafter the same wounds were examined at intervals of approximately seven days, for a period of five weeks.

The material of each swab was inoculated into meat water tubes—"a" and "b." The tubes were boiled prior to inoculation, in order to ensure anaërobiosis.

Tube "a" was incubated anaërobically to obtain a growth of non-sporulating organisms.

Tube "b" was heated to 65° C. for 30 minutes before incubation, in order to obtain growths of sporing anaërobes only.

Each culture was examined ten times at intervals of three or four days.

When the anaërobes developed rapidly, and in some variety, the swab was marked in the records as "heavily infected"; and when the cultures showed only a slight or medium growth, the result was noted as "light anaërobe infection."

It might be argued that a notation of this kind is arbitrary, but only by taking a broad view, could one preserve a correct perspective of the results obtained. In Diagrams XIII, XIV, XV, XVI and XVII, pp. 191-195, are given the details of the examination of each swab.

(iii) When organisms, having the morphological characters of *B. tetani*, appeared in the meat cultures, these were sub-cultured into the "exhausted" medium described in previous communications, and the growth therefrom tested by the agglutination methods. When a culture was obtained that agglutinated in the presence of any of the type sera, its toxogenicity was then tested by sub-culture from the original meat tube, and, in certain instances, its infective quality was also examined.

(iv) The tubes "a," incubated without previous heating, were examined with a view to demonstrating the presence of *B. Welchii*.

*Note.* This procedure was only introduced after swabs from 36 cases had already been examined. The figures relating to *B. Welchii* deal, therefore, with only 64 cases.

It is specially to be noted, that these swabs were always taken by one observer and we wish to record our debt of gratitude to Miss Smithwhite who was in charge of this section of the work. Her enthusiasm and care in collecting material, the attention devoted to keeping detailed and accurate records of each case, and the extracting of the necessary information from the field cards of the cases investigated—duty demanding much expenditure of time and labour—have greatly assisted in the prosecution of the work.

(b) *Inherent fallacies incidental to all bacteriological inquiries dealing with the treatment of wounds.*

In addition to the difficulties that are encountered in making an inquiry into the bacterial flora of wounds, the problem under consideration presented its own peculiar difficulties; of these the following may be cited.

(i) The diversity of methods of treatment in use both in France and in England is such that, to obtain a sufficiency of observations on which to base definite conclusions concerning any one method, would involve not months, but years of study.

(ii) It is well nigh impossible to get continuity of method in France and in England.

(iii) While a certain number of cases are evacuated to England, and find their way to hospitals in a single district in this country within a week or two after the soldiers have been wounded, the majority of wounds are not seen on this side until considerably later than this. Herein arises an almost insuperable difficulty, viz. that men whose wounds are slow to heal are sent to England, with the result that a special type of wound, showing chronic infection with a variety of organisms, both anaërobic and aërobic, is liable to predominate in a series of wounds examined in home hospitals.

(iv) Apart from these influences, there is the fact, that owing to idiosyncrasy of certain of the patients, some wounds will heal, not because of, but one might almost say in spite of, treatment; whereas others, apparently comparable to them, cannot be induced to heal under any treatment. The two cardinal points, which must not be lost sight of, are:

(a) That surgery is more an art than a science, therefore more depends upon the surgeon in charge of the case than on the dressing which he employs.

(b) That the best protection which the tissues can have against bacterial invasion due to any micro-organism, is the development of a layer of compact, healthy granulations on the raw surfaces.

Bearing these facts in mind, one could not hope to show any startling effects produced by the employment of one procedure, or of any series of procedures; so that the results given herein must be critically interpreted.

The following questions naturally arise:

(1) Does the presence of anaërobes in wounds—the presence of anaërobes in general, not the presence of certain species of anaërobes—seriously interfere with the healing of a wound?

(2) Is there any particular method of treatment commonly in use in France which leads, either to elimination of anaërobic infection or to rapid healing?

(3) Is there any method in use in home hospitals which is especially useful in attaining these ends?

(4) Is there any one surgical procedure, apart from the dressings

used, which is followed by special rapidity of repair or by elimination of anaërobes?

The problem under investigation had therefore to be examined from such standpoints, that these questions might be answered as far as possible.

(c) *Does the presence of anaërobes in wounds seriously interfere with the process of healing?*

In dealing with a series of cases in which swabs were taken seven days before the wound had healed sufficiently for the patient to be considered convalescent, the following results were obtained:

- (a) No anaërobes were found in 19 instances.
- (b) Light anaërobe infection was present in 20 instances.
- (c) Heavy anaërobe infection was present in four instances.
- (d) Tetanus bacilli were demonstrated in the swabs in (c) in three instances.

Of 43 cases which failed to heal during the period over which the investigation extended—each wound was examined for five weeks at intervals of one week—the swab taken during the last week in which the patient was under examination yielded:

- (a) No anaërobes in 29 instances.
- (b) Light anaërobic infection in 14 instances.
- (c) Tetanus bacilli were present in one instance.

These figures indicate that anaërobic infection may persist in wounds up to the time of healing, and that the presence of such infection does not seriously retard the process of repair.

It must not be assumed from this statement, that an anaërobic infection is, in the opinion of the writers, of little or no importance in the pathology of the later phase of wound infection. The time, however, during which the presence of anaërobic bacilli is of special significance, is the first day or two after the injury has been received, and the variety, quite as much as the mass of the infection, is of importance at this time.

It must be appreciated too that, short of making a detailed inquiry into the flora of a large number of wounds, one cannot assume that the presence of anaërobes, even in the later phases of the process of repair, are of no significance; for the presence of bacilli of the *Vibrio septique* group, *B. Welchii* of *B. oedematiens*, and of *B. tetani* must always be a menace.

The detection however, of certain of these, is extremely difficult, and a special research would have to be devoted to the study of each, were a really satisfactory account to be given of the problem under consideration. Such detailed inquiry, in the present instance, has been therefore limited to the demonstration of *B. tetani* and of *B. Welchii*.

It is to be noted that in the case of *B. Welchii*, morphological characters were relied upon for demonstrating the presence of this bacillus—not a highly satisfactory method, but one which we were forced to adopt owing to the labour and expense which other methods would have involved.

(d) *Is there any method of treatment commonly used by the Army Surgeon abroad which tends to eliminate anaërobie infection?*

The wounds which were dealt with in the present series of examinations had, in the majority of instances, been treated either with flavine or with eusol, before removal to England. These wounds will therefore be grouped in three series:

- (a) Treated with flavine.
- (b) Treated with eusol or Carrel Dakin.
- (c) Treated by other methods.

In considering this question, it was decided to deal only with the wounds of men who arrived in England within ten weeks of the date of wounding. If such a period be not put to the type of case considered, the difficulty of assessing the value of any procedure employed is greatly increased, owing to the number of refractory cases which would be introduced into the series.

(a) *Cases treated with flavine before evacuation to England.*

Twenty-two cases of the present series were treated with flavine abroad, and arrived in England within ten weeks of the receipt of injury. From them 72 swabs were examined.

35 swabs showed no anaërobic infection

32 „ „ light „ „

5 „ „ heavy „ „

In none of these swabs was *B. tetani* found.

(b) *Cases treated with eusol or Carrel Dakin before evacuation to England.*

Of 78 swabs from 22 wounds treated with eusol abroad and sent to England within the ten-week period,

26 swabs failed to give growth of anaërobes

31 „ gave light „ „

21 „ „ heavy „ „

*B. tetani* appeared in eight swabs from six wounds in this series.

(c) *Cases treated by various methods.*

There remained 28 cases examined with the ten-week period comprising

10 treated with B.I.P.P.

7 „ dry dressings,

7 „ boric foment,

4 „ saline or salt packs.

I shall consider these together, as the number of each is too small to justify their being considered separately.

Ninety-two observations were made on the 28 wounds, and the following results were obtained:

42 showed no growth of anaërobes,

39 „ light „ „

11 „ heavy „ „

*B. tetani* was grown from seven swabs obtained from five different wounds. Diagram VII shows these results expressed as percentages.

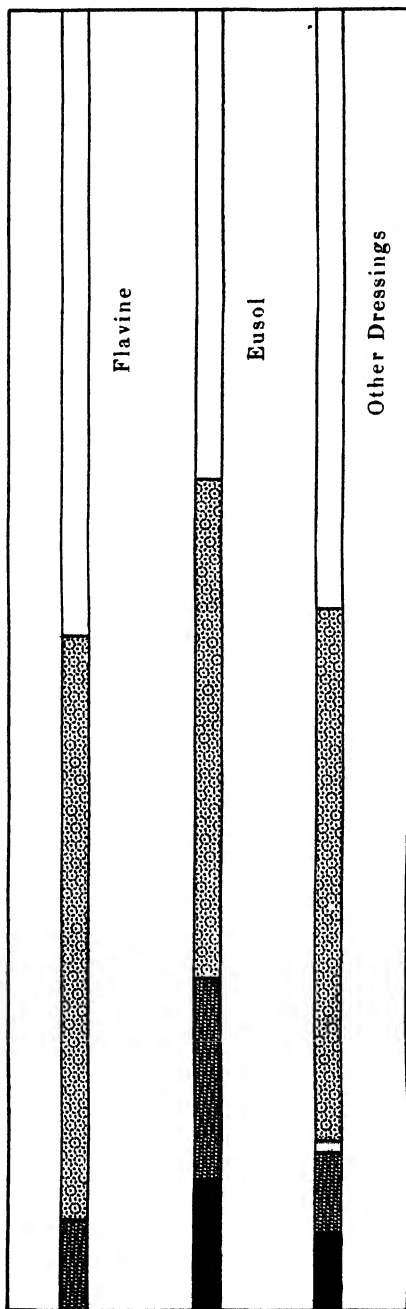


DIAGRAM VII. Influence of dressings used abroad on anaerobe infection of wounds. Results expressed as percentages of swabs examined.

■ = Tetani + heavy anaerobe infection.    ▨ = Tetani + light anaerobe infection.  
 ▩ = Heavy anaerobe infection.    ▧ = Light anaerobe infection.  
 □ = No anaerobe infection.

The dark columns refer to heavy anaërobe infection, the light to light anaërobe infection. The black columns indicate that *B. tetani* was found along with heavy infections of other anaërobes, and the hatched column that *B. tetani* was present with light concomitant anaërobe infection.

It is not easy to comment on the results shown in the above diagram, for they may well give a false impression, owing to the fact that 11 of the wounds in the flavine series had been excised, while only six of those of the eusol series, and five of those in which other dressings had been used, were so treated.

Both the low rate of "heavy anaërobe infection" and the absence of tetanus bacilli in those wounds originally treated by flavine, appear to suggest that that reagent is suitable for primary dressings. The results may, however, mean that excision of the wounds was, in the instances quoted, the most important factor in eliminating infection.

This subject will be again considered in dealing with the "time factor" in the healing of excised wounds, as compared with that of non-excised wounds.

In Diagrams XIII and XIV (pp. 191, 192) are graphically shown the results of each examination of the swabs dealt with above.

(e) *Influence of dressings in use in the Home Hospitals on the degree and persistence of anaërobic infection of wounds.*

In this series, the arbitrary time limit of ten weeks was again adhered to, in order that the results obtained might be, as far as possible, comparable with those dealing with the methods used abroad.

The series comprises an additional three cases in which the treatment abroad was not recorded on the field cards, so that 75 in place of 72 cases are herein considered.

(a) *Cases treated by boric fomentations.*

It was surprising to find that boric fomentation was the favourite method of treatment in the hospitals from which the material was obtained, and of cases so treated there were 35 involving 123 observations.

55 swabs failed to give growth of anaërobes,

49 „ gave light „ „

19 „ „ heavy „ „

*B. tetani* was obtained from 11 swabs in seven cases.

(b) *Cases treated by eusol.*

Of cases treated by eusol or the Carrel Dakin method there are 22, involving 58 observations.

27 showed no anaërobe infection,

23 „ light „ „

8 „ heavy „ „

*B. tetani* was obtained from three swabs in three cases.

*(c) Cases treated by other methods.*

Eighteen cases of the series were treated by other methods (comprising 13 in which saline was used), in which a variety of procedures had been employed.

These involved 71 observations, giving the following results.

30	swabs failed to give growth of anaërobes,
30	„ showed light anaërobe infection,
11	„ heavy „ „

*B. tetani* was found in three instances from three cases.

These results are summarised in Diagram VIII, p. 180.

These results are instructive, for they show in a remarkable manner, that no particular dressing can be recommended as likely to produce marked diminution in the anaërobe flora of wounds during the process of repair.

Experimental evidence bears this out—thus, Brilliant Green, which in certain low concentrations will markedly inhibit the growth of bacteria, including the anaërobes, in broth or in serum, is much reduced in its inhibitory activity if a piece of fresh living tissue be added to the cultures.

The conditions obtaining in cultures in fluid media enriched by the addition of fresh tissue, more closely approximate the conditions of a wound, than do cultures which are not so enriched.

It is probable that this inhibition of antiseptic activity demonstrated in culture tubes is multiplied manifold in wounds, owing to the large surface of granulation to which the reagent is exposed.

All that can be hoped for then, from the use of any antiseptic so far employed, is, that it will reduce mass infection of wound exudates; it will not, however, eliminate infection.

As a mild degree of anaërobe infection is quite compatible, so far as can be seen, with unretarded progress of healing; and, as we cannot hope to eliminate the anaërobes by the use of any antiseptic, the question at issue must be dealt with from another point of view.

The details of the examination of each swab are given in Diagrams XV and XVI (pp. 193, 194) at the end of this section.

*(f) Rapidity with which healing takes place under various dressings.*

So long as a wound remains open it is, to a greater or less degree, susceptible to bacterial invasion of all kinds. If then, any particular procedure can be shown to cause rapid healing of wounds, such is to be recommended, both from the standpoint of surgery and from that of economics.

Three points of view have to be considered in dealing with this question.

(a) The nature of the dressing used abroad.

(b) The nature of the dressing in home hospitals.

(c) The wounds treated by excision must be contrasted with those that have not been excised.



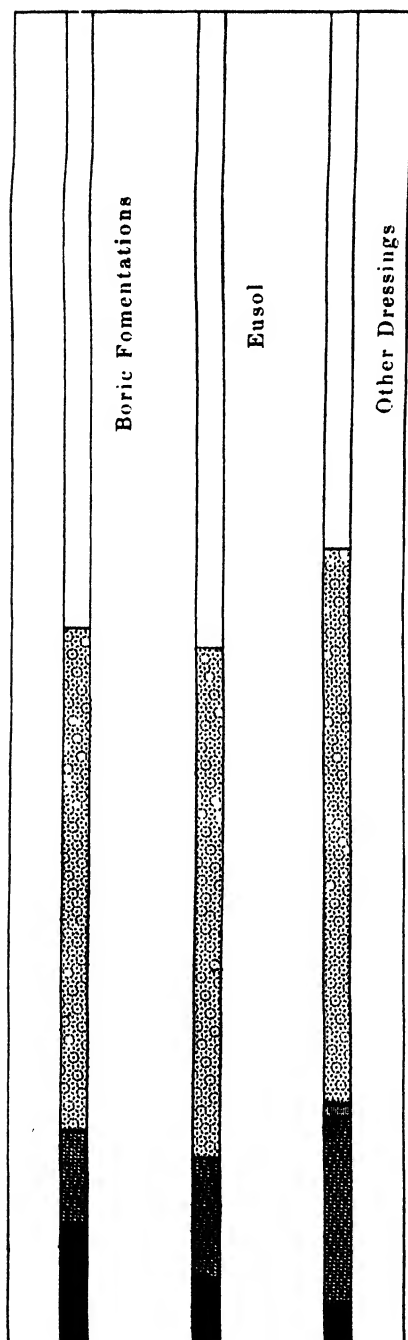


DIAGRAM VIII. Influence of dressings used in Home Hospitals on anaerobe infection. Results expressed as percentages of swabs examined.

- = Tetani + heavy anaerobe infection.
- = Tetani + light anaerobe infection.
- = Heavy anaerobe infection.
- = Light anaerobe infection.
- = No anaerobe infection.

(f') *Influence of dressings used abroad upon the rate of healing.*

1. *Flavine.*

Twenty-one wounds fulfilling the conditions arbitrarily laid down concerning evacuation to England within a ten-week period, were treated with flavine abroad. Of these 14 recovered, the recoveries being distributed thus:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	2	2
2 weeks	8	6
3 "	4	4
4 "	2	0
5 "	1	0
6 "	1	0
7 "	1	0
8 "	0	0
9 "	2	2
10 "	0	0

2. *Eusol.*

Twenty-two wounds receiving early treatment with this reagent behaved as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	3	2
2 weeks	3	1
3 "	3	1
4 "	2	1
5 "	3	2
6 "	2	0
7 "	1	0
8 "	1	1
9 "	1	0
10 "	3	2

3. *Other methods.*

In the case of 26 wounds which had been treated abroad by other methods, the following results were obtained:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	4	3
2 weeks	8	5
3 "	5	1
4 "	2	2
5 "	0	0
6 "	1	0
7 "	2	0
8 "	2	2
9 "	0	0
10 "	2	0

These results are summarised in Diagram IX, in which both the recovery rate and the time when recovery occurred are graphically shown. For the purposes of comparison the figures are reduced to a common denominator.

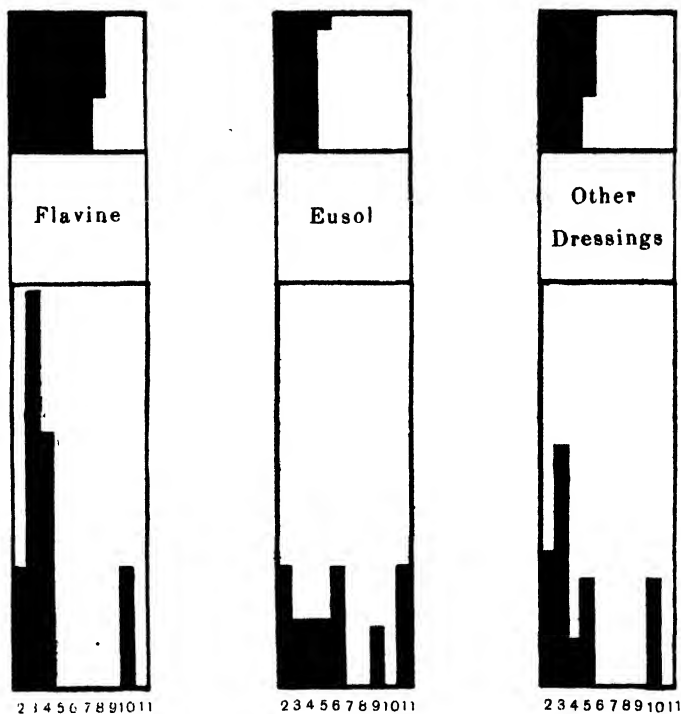


DIAGRAM IX. The upper portion of the diagram indicates the percentage recovery with each dressing. The lower indicates the wounds which recovered. Figures relate to week in which recovered cases were first examined in England. An arbitrary time limit of five weeks from first examination was taken as period for recovery to take place.

These results appear to indicate that flavine is the most valuable dressing of those considered in the present series, both as regards the number of recoveries and the rapidity of recovery.

It must be noted, however, that there is a preponderance of excised wounds in the flavine series.

#### (f'') *Influence of dressings used in Home Hospitals.*

When considered from the standpoint of dressings used in Home Hospitals, the following results appear:

##### 1. *Boric fomentations.*

Thirty-six wounds were treated by boric fomentations, with 12 recoveries, distributed as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	5	2
2 weeks	7	4
3 ..	7	3
4 ..	3	1
5 ..	3	0
6 ..	2	0
7 ..	3	0
8 ..	0	0
9 ..	6	2
10 ..	0	0

### 2. *Eusol.*

Twenty-two wounds were treated with eusol, giving 15 recoveries, distributed as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	4	4
2 weeks	5	4
3 ..	3	2
4 ..	1	1
5 ..	2	1
6 ..	1	1
7 ..	4	2
8 ..	0	0
9 ..	0	0
10 ..	1	0

### 3. *Other methods.*

Eighteen wounds were treated by other methods—with nine recoveries, distributed as follows:

Patients examined in England within	Number of wounds examined	Number which healed
1 week	0	0
2 weeks	5	4
3 ..	8	2
4 ..	2	1
5 ..	1	1
6 ..	0	0
7 ..	1	1
8 ..	0	0
9 ..	0	0
10 ..	1	0

*Note.* Of the series treated by boric fomentations four had been excised. Of those treated with eusol nine and of those by other methods three.

These results are summarised in Diagram X, p. 184, which shows graphically both the recovery rate and the time when recovery took place.

The results summarised in Diagram X suggest that the cases treated with eusol in Home Hospitals heal more rapidly than do those treated by other methods. Here again, however, there is seen a preponderance of the excised wounds in the series.

The results then do not give information as to which dressings of those examined could be recommended for use in England, as being of special value in stimulating healing.

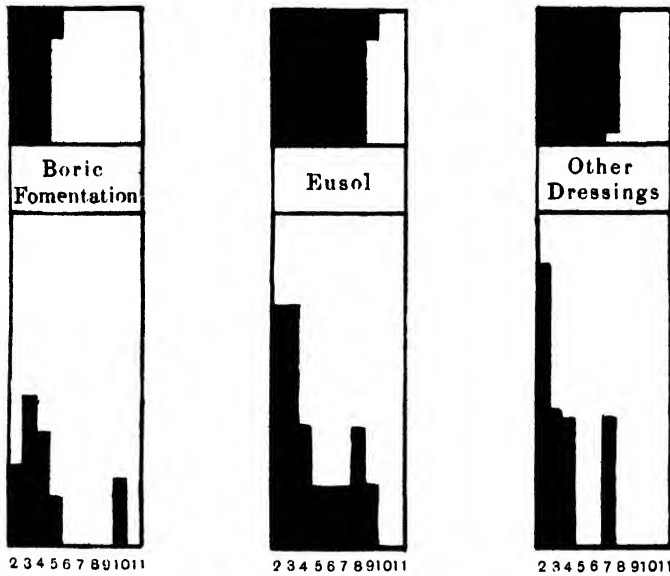


DIAGRAM X. The upper portion of the diagram indicates the percentage recovery with each dressing. The lower indicates the wounds which recovered. Figures relate to week in which recovered cases were first examined in England. An arbitrary time limit of five weeks from first examination was taken as period for recovery to take place.

(g) *Influence of excision.*

*Is there any one surgical procedure, apart from the dressings employed, which leads to the rapid healing of wounds?*

If excised wounds be contrasted with those not so treated, it is seen that within the arbitrary period of ten weeks, 16 out of 26 excised wounds had progressed sufficiently for the patients to be considered convalescent. While of the non-excised wounds, 51 in number, 24 became convalescent. These findings are summarised in Diagram XI, in which are indicated both the recovery rate, and the time within which recovery occurred.

This diagram indicates:

(a) That the recovery rate is higher in the *excised* than in the *non-excised* wounds.

(b) That the distribution of the recoveries in point of time is more uniform in the *excised* than in the *non-excised* wounds. This latter point appears to the writers to be a matter of some importance, as it suggests, that a more diverse type of wound heals within a given period after excision, than is the case when excision is not practised. This really means, that in the

case of the *non-excised* wounds, unless healing occurs within the first three or four weeks, the convalescence may be protracted.

The details of the examination of swabs from *excised wounds*, irrespective of the dressing used, are shown graphically in Diagram XVII, p. 195.

Before proceeding to the next sub-section of the investigation, dealing with wounds, the examination of which was commenced after the expiry of

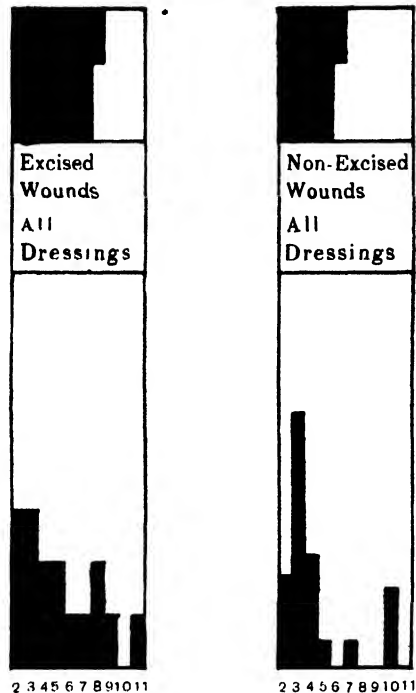


DIAGRAM XI. The upper portion of the diagram indicates the percentage recovery in each instant. The lower indicates those wounds which recovered. Figures relate to week in which recovered cases were first examined in England. An arbitrary time limit of five weeks from first examination was taken as period for recovery to take place.

the ten-week period, the question of the influence which anaërobes have upon healing, and also the influence which various dressings exert upon anaërobe infection, may be summarised thus:

(i) A mild degree of anaërobe infection does not prevent, and does not apparently even retard, the process of healing.

(ii) Wounds treated with flavine in France have, on the whole, a lesser degree of infection with anaërobes, than those treated by other methods. The fallacy due to the preponderance of *excised* wounds so treated, renders it difficult to offer an opinion as to whether flavine or excision has been the factor producing the desired result.

(iii) None of the treatments used in home hospitals, which we were in a position to investigate, eliminate anaërobe infection with especial rapidity.

(iv) As regards the rapidity with which the wounds healed, the best results were obtained in those treated with flavine abroad and with eusol in England. It is to be noted that a preponderance of *excised* wounds in both instances renders difficult the assessment of these results.

(v) On contrasting *excised* with *non-excised* wounds, it is found, that the recovery rate is higher and more uniform in wounds which have been *excised* than in those which have not been so treated.

(h) *Examination of wounds of long standing.*

In addition to the series that has already been considered, 12 wounds were examined in men who arrived in England between the 10th and 15th weeks after reception of the injury. This involved the examination of 37 swabs.

No anaërobes found in 23 instances,

Light anaërobic infection in 10 instances,

Heavy „ „ 4 „

*B. tetani* was found in seven swabs from five cases.

Finally, as a matter of scientific interest, it was decided to examine some wounds of very long standing.

(a) Wound examined during the 17th and 18th weeks gave one swab which showed a heavy growth, and one which failed to give growth of anaërobes.

(b) Wound examined from the 18th to the 23rd week gave three swabs producing a heavy growth of anaërobes, and two which showed no growth of such organisms.

(c) Wound examined from the 22nd to the 25th week gave two swabs showing heavy anaërobic infection, one with light infection, while in two anaërobes failed to grow.

(d) Wound examined from the 23rd to the 27th week gave three swabs with light anaërobe infection and two with none.

(e) Wound examined from the 30th to the 33rd week gave no growth of anaërobes.

(f) Wound examined during the 32nd and 33rd weeks gave one swab with a light anaërobe infection and one showed no anaërobe infection.

(g) Wound examined from the 35th to the 38th week, one swab showed heavy growth of anaërobes and three showed no growth of anaërobes.

(h) Wound examined from the 37th to the 40th week all four swabs failed to give growth of anaërobes.

(i) Wound examined from the 38th to the 41st week gave four successive swabs showing heavy anaërobe infection.

(j) Wound examined from the 42nd to the 45th week gave three swabs showing light anaërobe infection and one showing none.

(k) Wound examined from the 43rd to the 47th week gave five successive swabs containing *B. tetani*, three being accompanied by heavy and two with light anaërobie infection.

(l) Wound examined from the 44th to the 47th week gave one swab with light anaërobie infection and three with none.

If then the results obtained in examining wounds between the 15th and 50th weeks after receipt of injury be summarised, the following results are obtained:

24 wounds were examined involving the examination of 82 swabs.

In 46 instances no anaërobes were found,

„ 22 „ light anaërobie infection was found,

„ 14 „ heavy „ „ „ „

*B. tetani* was obtained in 12 swabs from six cases.

On six occasions *B. tetani* was found along with heavy anaërobie infection and on the other occasions with light anaërobie infection.

It seems at first sight remarkable that these results are so bad, and compare unfavourably with any that have gone before, but the wounds considered are practically picked refractory cases and therefore occupy a category by themselves.

There are, in addition, two especially interesting observations:

(i) Five swabs were taken from a case between 104th and 108th weeks from date of wounding, three out of the five swabs were heavily infected with anaërobes, two out of the three containing *B. tetani*.

(ii) Five swabs were also taken from a case between the 106th and 130th weeks after reception of the injury, two gave a heavy growth of anaërobes *B. tetani* being present in large numbers in the cultures, one gave a light growth of anaërobes, and two gave no growth of these organisms.

These observations call attention to the remarkably long periods during which *B. tetani* and other anaërobes may persist in wounds. The importance of the observations is, that they indicate how necessary it is to administer a prophylactic dose of antitoxin the day before any operation for cosmetic or other purpose is performed at the site of an old wound. It would be advisable, too, to give, if possible, anti-gas-gangrene serum along with the prophylactic tetanus antitoxic serum.

(i) *Examination for presence of B. tetani in a series of 100 wounds.*

The tetanus bacilli obtained from 100 consecutive wounds of men showing no evidence of tetanus are tabulated on p. 188.

Commenting on these results, it is seen, that 14 cultures elaborating spasm-producing toxin were obtained from 100 consecutive wounds. In carrying out the toxin tests 0.2 c.c. was the largest quantity employed, when mice were the experimental animals used.

In four instances, while the toxin experiments were negative, typical



No.	Animal experiment	Agglutination	Since wounding	Where wounded
1.	Toxin experiment positive	Type I	7 days	Ypres
2.	" " "	"	7 "	Ypres
3.	" " "	"	22 "	Ypres
4.	" " negative	"	24 "	Albert
	Infection experiment positive			
5.	Toxin experiment "	"	25 "	Villers Bretonneux
6.	" " "	"	30 "	Meri-court
7.	" " negative	"	32 "	Cambrai
	Infection experiment positive			
8.	Toxin experiment negative	"	35 "	Cambrai
	Infection experiment positive			
9.	Toxin experiment negative	"	39 "	Cambrai
	Infection experiment positive	"	39 "	Cambrai
10.	Toxin experiment "	"	64 "	Battersea
11.	" " "	"	112 "	Ypres
12.	" " "	"	130 "	Cambrai
13.	See Footnote 1	"	733 "	Hohenzollern Re-doubt
14.	Toxin experiment positive	"	860 "	Poelcapelle
15.	" " "	"	882 "	Cambrai
16.	" " "	Type II	15 "	Bapaume
17.	" " "	"	138 "	Passchendaele
18.	See Footnote 2	"	100 "	Cambrai
19.	" " "	"	54 "	Monchy
20.	Toxin experiment positive	Type III	16 "	Orvillers
21.	" " "	Type IV	305 "	Hermies

Footnote 1. With reference to No. 13 toxin experiments were negative, and when an attempt was made to carry out the infection experiment, the animals died from gas gangrene.

Footnote 2. Cultures 18 and 19. Culture 18, which agglutinated in presence of Type II serum, was lost before the animal experiments were completed. Culture 19 is still under observation.

tetanus infection could be produced when washed cultures were injected together with a tissue debilitant. Therefore, in at least 18 per cent. of the present series of wounds, tetanus bacilli could be recovered.

This figure is considerably higher than any previous investigations would have led one to expect. The reason for this somewhat high figure is, that unless wounds be repeatedly examined, tetanus bacilli if present in small numbers may be missed; just as throat swabs, in cases of diphtheria, may give negative results owing to the operator having failed to swab that area of the throat in which *B. diphtheriae* is present.

Diagrams XIII, XIV, XV, XVI and XVII which give the result of the examination of each swab graphically illustrate this point.

(j) *Examination for presence of organisms having the morphological characters of B. Welchii in a series of 100 wounds.*

In the series of wounds under consideration the presence of organisms having the morphological appearance of *B. Welchii* was also noted, and an attempt was made to determine whether any of the dressings commonly

used, either at home or abroad, tended rapidly to eliminate this organism from wound exudates.

From this inquiry, the following conclusion was drawn: "That no dressing among those investigated could be especially recommended as likely to produce rapid elimination of *B. Welchii*."

As the details of the examination constitute what is virtually a repetition of the findings already set forth in connection with the inquiry into the influence which various dressings exert upon the reduction of all anaërobic infections, they will not be dealt with *in extenso*.

The results of the examinations call attention, however, to a marked difference between *excised* and *non-excised* wounds in this connection. While there is but little difference between the percentages of *excised* and *non-excised* wounds, the exudates of which contain *B. Welchii*, there is a marked difference between the two classes in respect of the period over which infection with *B. Welchii* can be demonstrated. This period is much shorter in the case of *excised* wounds than in the case of *non-excised* wounds.

The actual findings are as follows:

On a series of 100 wounds 46 were found to contain *B. Welchii*.

(a) In four wounds—two excised, two non-excised—*B. Welchii* disappeared before the second week.

(b) In eight wounds—five excised, three non-excised—*B. Welchii* disappeared before the third week.

(c) In six wounds—one excised, five non-excised—*B. Welchii* disappeared before the fourth week.

(d) In three non-excised wounds, it disappeared before the fifth week.

(e) In four wounds—three excised, one non-excised—it disappeared before the sixth week.

(f) In two wounds—one excised, one non-excised—it disappeared before the seventh week.

(g) Thereafter, only non-excised wounds contained *B. Welchii*. In one this organism disappeared before the 8th week, three before the 9th week, one before the 11th, four before the 13th, one before the 15th, one before the 16th, two before the 17th; and five wounds were shown to contain *B. Welchii* between the 27th and 47th weeks after infliction of the injury. These facts are set forth diagrammatically in the following figure.

*Note.* Attention is called to the fact, that in many of the instances quoted above, the number of organisms present in the cultures which had the morphological appearance of *B. Welchii* was small. It is probable that, without careful and repeated examination of the growths, the presence of this bacillus would not have been appreciated.

These findings are in full agreement with those which were obtained in making inquiry into the influence which various dressings and surgical procedures exert upon all anaërobic infections of wounds.

They are especially valuable, in that they show fairly conclusively, "*that excision does not eliminate infection with the anaërobic bacilli but it removes*

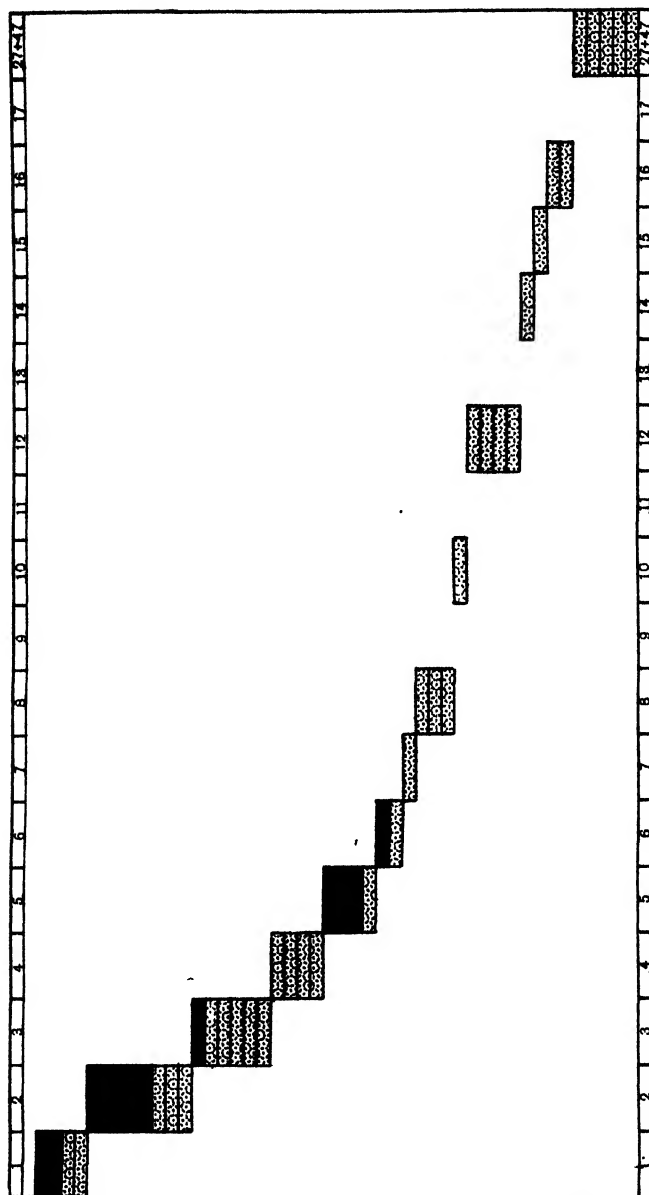


DIAGRAM XII. This figure shows the number of wounds in which *B. Welchii* was present and indicates the last swab in which it could be demonstrated in each instance.

■ = In excised wounds.

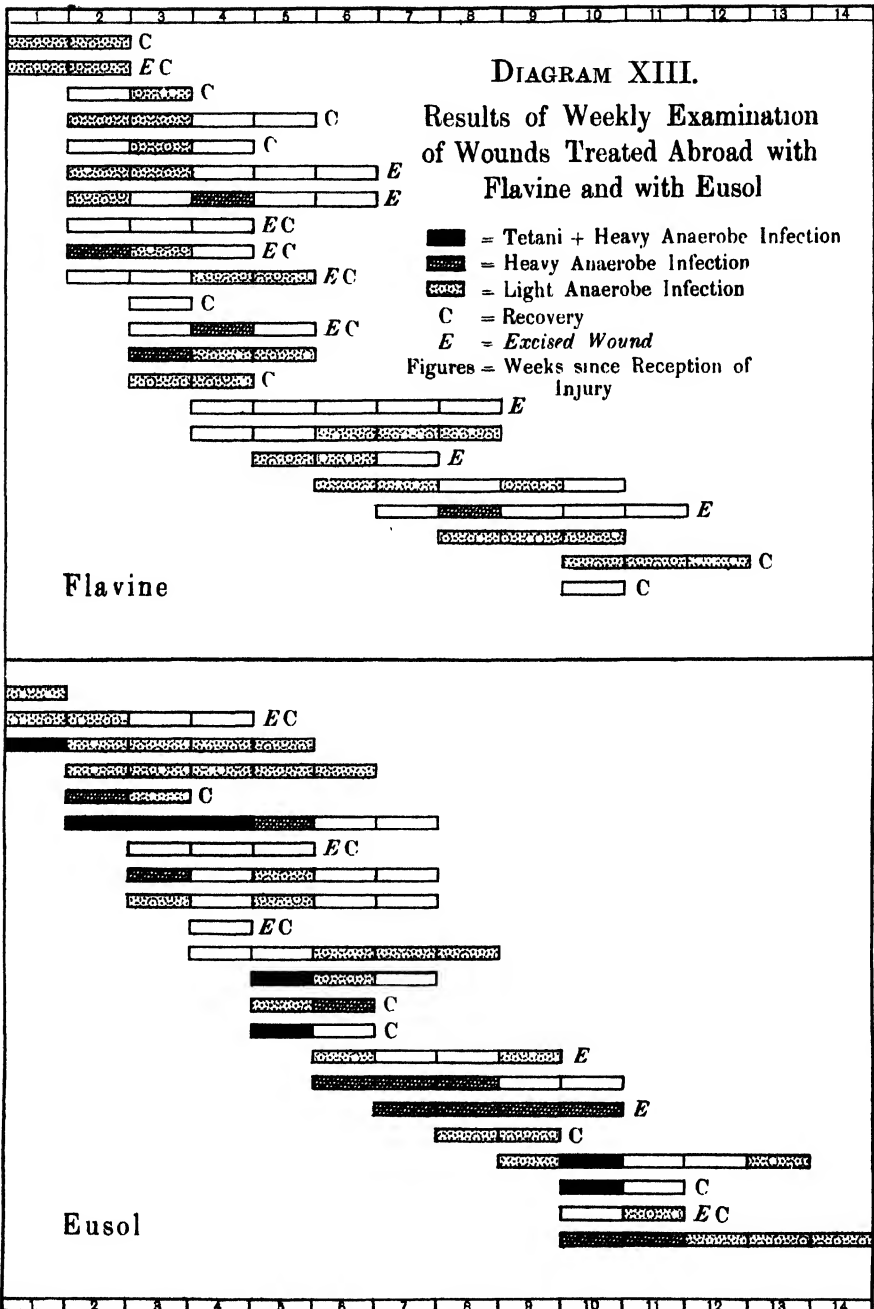
▨ = In non-excised wounds.

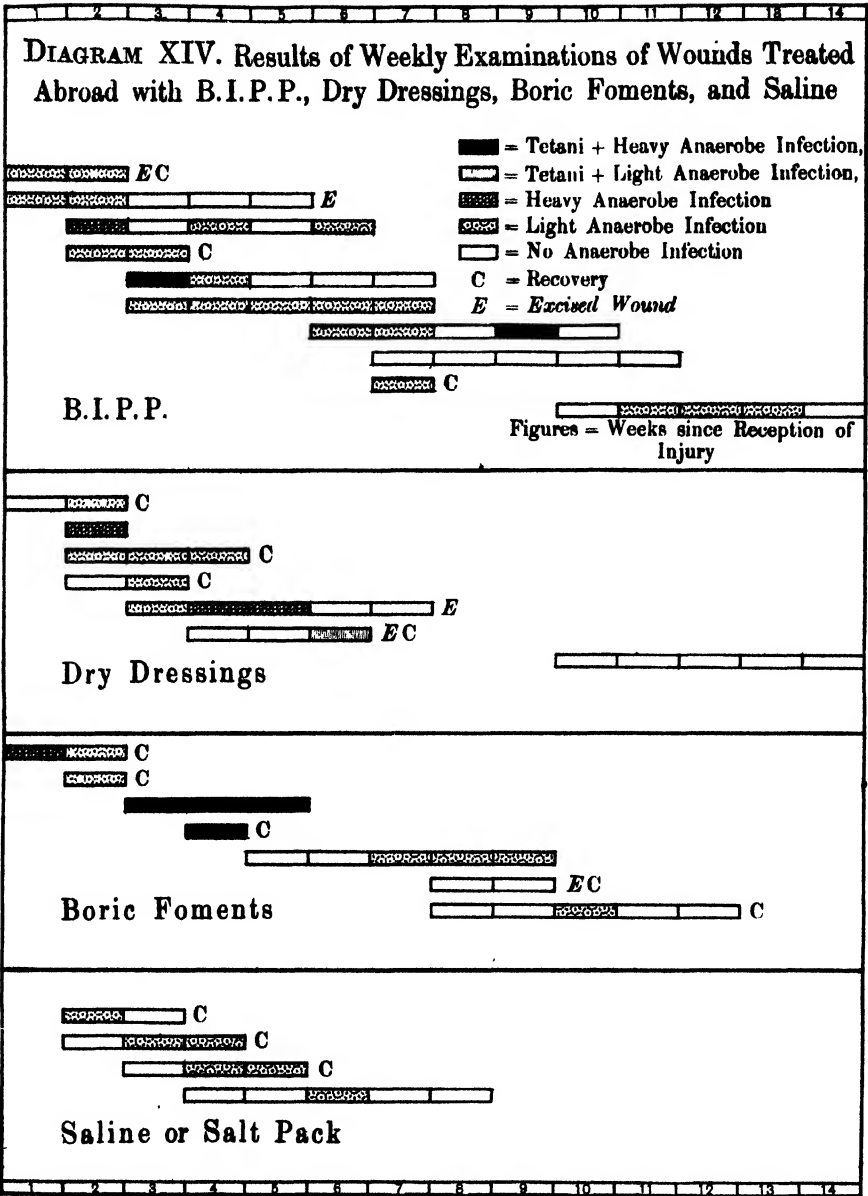
Figures = Weeks since reception of injury.

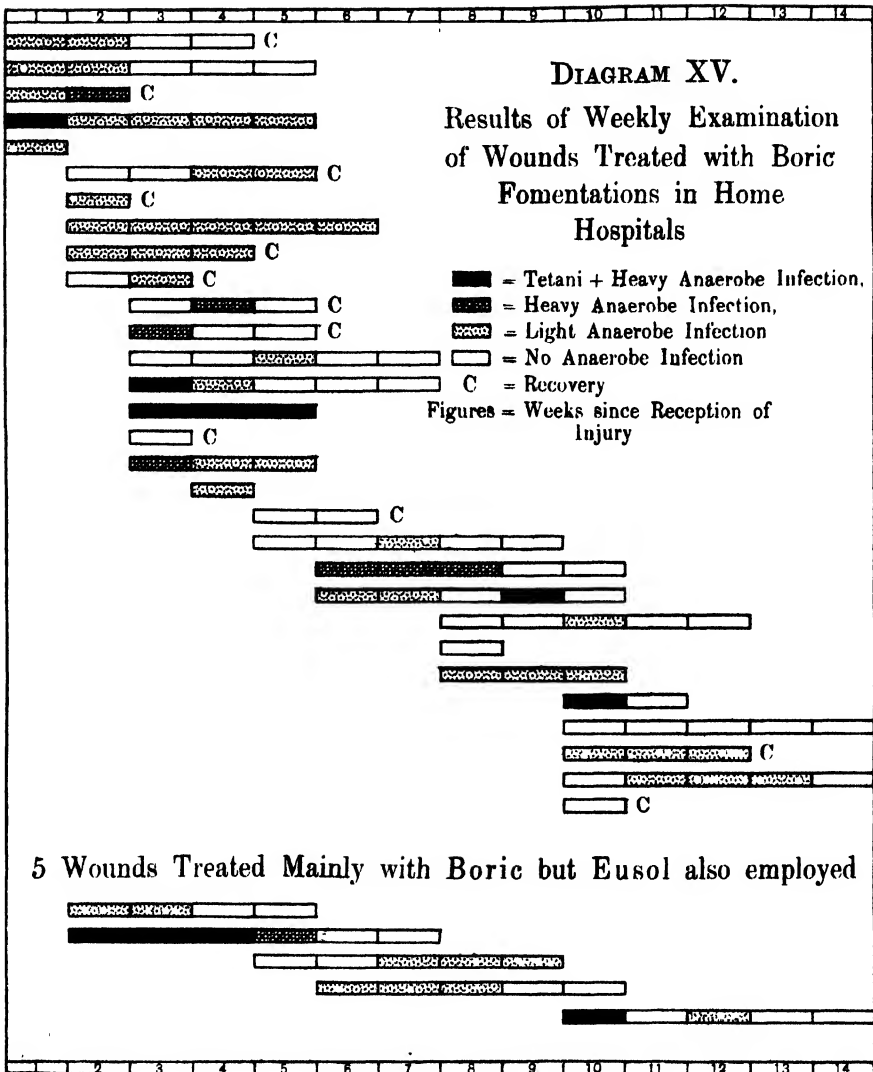
Total number of excised wounds = 27, *Welchii* present in 12 = 44 %.

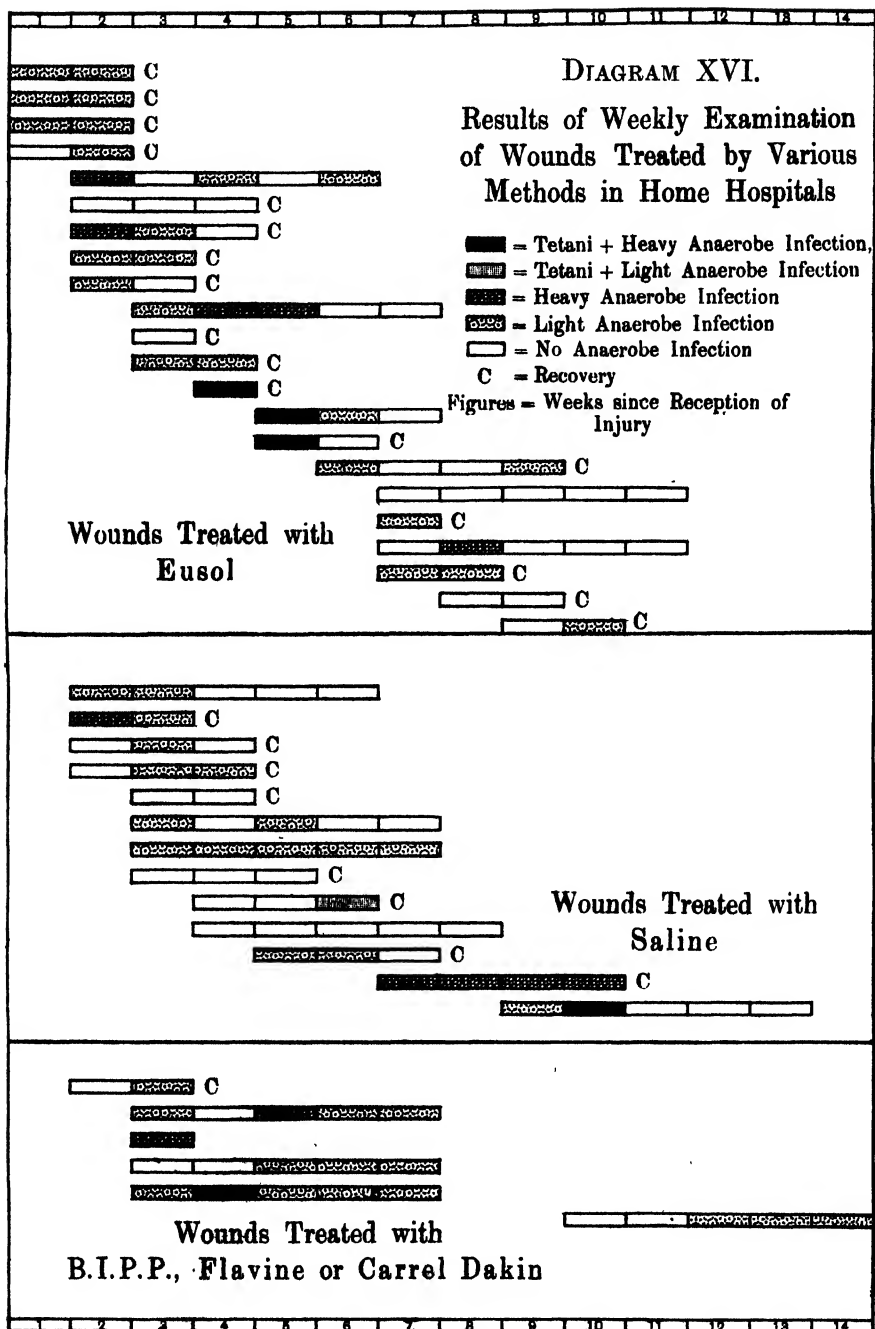
Total number of non-excised wounds = 79, *Welchii* present in 34 = 46 %.

*those conditions which enhance the danger arising from the presence of these organisms."*



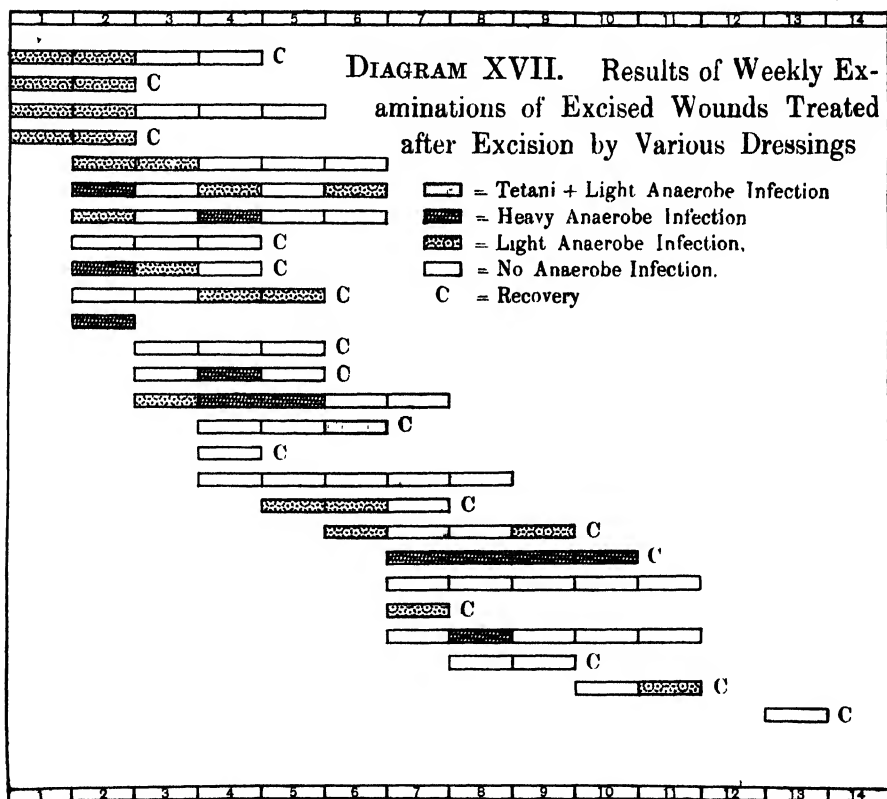






## CONCLUSIONS FROM SECTION VI, pp. 172-195.

1. No one dressing, of those dealt with in the present investigation, can be especially recommended as being valuable for the elimination of anaërobic micro-organisms in general from wound exudates.
2. Neither can any one dressing be recommended as especially valuable in eliminating *B. tetani* or *B. Welchii*.
3. The presence of a mild degree of anaërobe infection in wounds does not necessarily prevent, or even retard, the process of healing.
4. Excised wounds heal more rapidly than do non-excised wounds, and therefore by excision the danger period is reduced during which infection with either *B. tetani* or the gas gangrene bacilli may be a menace.





## SECTION VII.

CAN AGGLUTINATION OF STOCK EMULSIONS OF *B. TETANI* BY THE SERUM OF PATIENTS SUFFERING FROM EARLY TETANUS BE EMPLOYED AS A METHOD FOR DIAGNOSIS AND FOR DETERMINING "TYPE OF INFECTION" IN TETANUS?

Serum from over 60 cases of tetanus was used for agglutinating stock emulsions of the 4 Type bacilli. At the same time a number of normal sera were tested in the same way.

The result showed that certain normal sera may agglutinate tetanus bacilli of one or other Type in dilutions up to 1/80. None of the sera from cases of the disease could be definitely demonstrated to be of much higher titre; and further, in those cases in which the infecting organism was typed, no relationship could be shown between the Type of the infecting organism and the agglutination reaction performed with the patient's serum. One had to conclude, therefore, that agglutination could not be employed for making a "preclinical" diagnosis of tetanus, nor could it be used for determining to which serological Type of the bacillus the organism belonged which was responsible for the causation of the disease in each case.

## SECTION VIII.

RELATION OF SEROLOGICAL TYPE OF BACILLUS ISOLATED, TO HAEMAGGLUTINATING TYPE OF MEN FROM WHOM THE BACILLUS WAS OBTAINED.

As a matter of scientific interest, the haemagglutinating property of these sera was, where possible, determined. The reason for undertaking this inquiry was, that it might be suggested, that the serological Type of the organism depended upon its being parasitic on individuals belonging to a particular haemagglutinating group of men; for instance, the Type I bacillus might evolve as a result of its being parasitic upon an individual of haemagglutinin Group II. Type III bacillus might result from parasitism on an individual belonging to another haemagglutinating group, etc. Such a relationship could not be demonstrated; for example, of four individuals from whom Type III bacilli were isolated, two belonged to haemagglutinin Group II, one to Group III, and one to Group IV.

## SECTION IX.

A NOTE ON TWO CASES OF ABDOMINAL TETANUS.

The two following cases are of especial interest, in that tetanus developed after the performance of an abdominal operation, and the source of infection in each case was undoubtedly the intestine.

*Case I.*

A man, who had been wounded in France one year previously, was taken ill with appendicitis; some days after the operation tetanus developed with a fatal result.

Examination of material from the wound in the abdominal wall, from the stump of the appendix, and from the faeces in the descending colon, all yielded a growth of tetanus bacilli of serological Type I and all virulent for animals.

*Case II.*

In this case, which was forwarded to me unofficially, the victim of the disease was a girl of 4½. The history of the case was as follows:

Patient was admitted suffering from intussusception—a laparotomy was performed the same day and the intussusception was reduced. Five days later tetanus developed and terminated fatally. Material from the wound and faeces were both submitted for examination. Type III tetanus bacilli were found in both.

The main interest of the second case is that as the gut had not been opened, therefore the bacillus must have been in the gut, and the cause of the *infection* was probably the tissue debility produced by the intussusception.

## SECTION X.

PRESENCE OF *B. TETANI* IN THE FAECES OF MEN RETURNED FROM OVERSEAS COMPARED WITH THOSE FROM CIVILIAN FAECES.

Up to the present 21 specimens of faeces from men returned from overseas have been examined, and in seven instances organisms were obtained which agglutinated with one or other of the Type agglutinating sera. Of these, four were Type I bacilli, two were Type II and one was Type III. Of 31 specimens of faeces from civilians five gave cultures which agglutinated with Type sera. All proved to be Type I. It is to be noted that the investigation of these faeces was not so complete as was that of cultures obtained either from the wounds of men suffering from tetanus or from wounds of men showing no clinical evidence of tetanus.

The inquiry, therefore, makes no claim to accuracy, and the results obtained are mainly of academic interest.

The reason for conducting this inquiry was, that a number of cases of abdominal tetanus in soldiers had been reported to Major-General Sir David Bruce, K.C.B., F.R.S. The aggregate of these cases was considerable. Two explanations of their occurrence had to be considered:

(i) It might be that owing to the machinery for collecting information *re* tetanus being adequate, these cases were returned among those due to tetanus infection of war wounds. Seen together the number appeared to be

relatively large, but without consulting the Registrar General's returns, it could not be assumed that the number was larger than in times of peace.

(ii) It might be, on the other hand, that the conditions of active service leading to contamination of food stuffs, etc., with earth, resulted in sufficient unconscious geophagy to alter the flora of the gut, and to increase the number of bacillus tetani. The figures quoted are insufficient to demonstrate that the latter explanation is correct, and it is highly probable that the larger number of cases of abdominal tetanus among soldiers is apparent rather than real.

Nevertheless in view of the findings reported in this section, and in view also of the findings previously published by Pizzini, it might be advisable to err on the side of safety, and administer a prophylactic dose of antitoxin before performing an abdominal operation involving section of the gut, especially when such operations are performed on recently returned men.

### SYNOPSIS AND GENERAL CONCLUSIONS.

I. In Sections I, II, and III, are described the methods employed, and the results obtained, in making a systematic investigation of the prevalence of the various serological Types of *B. tetani*, both in wounds of men suffering from tetanus and in those of men showing no evidence of that disease.

This inquiry shows:

1. That the mortality among inoculated men from infection due to tetanus bacilli belonging to serological Type I is lower than that from infection with either Type II or Type III.

2. If the observations be limited to those cases in which the incubation period is 14 days or less, the mortality from infection due to Types II and III is higher than when Type I is the organism responsible for the causation of the disease.

3. If observations on the incidence of cases due to the various Types of *B. tetani* be considered from the same point of view, relatively more cases of Type II and Type III infection declare themselves within a period of 14 days than Type I.

4. From an unexpectedly large percentage—20 per cent.—of wounds of men who show no evidence of tetanus, *B. tetani* can be recovered at some period during the process of repair.

5. Of tetanus bacilli obtained from such "indifferent" wounds the majority conform to serological Type I.

These facts are susceptible of two explanations:

A. Either that Type I bacilli are, on the whole, less virulent, or less toxogenic, in character than are those of Types II and III;

B. Or, that the serum prophylaxis in use until recently, afforded more adequate protection against Type I infections than it did against infections due to Types II or III.

As all those cultures of *B. tetani* that I obtained from serum laboratories conformed to Type I on serological examination, it appeared that hypothesis B was worthy of serious consideration.

II. Section IV deals with experiments that were undertaken to demonstrate *in vitro* the stability of the Types after prolonged culture, and to investigate the presence of antibodies other than agglutinins in the sera of animals immunised by inoculating

- (a) the filtered products of growth—"Toxin,"
- (b) washed bacilli,
- (c) whole cultures.

The facts obtained from this section of the inquiry are:

1. That even when agglutinating sera of high titre are used the bacillary Types react specifically.

2. That even after very frequent sub-culture extending over a period exceeding one year, the bacilli remain true to type.

3. Anti-bacterial qualities, other than agglutinins, can be evoked by inoculating whole cultures of *B. tetani* into animals. For technical reasons the anti-bacterial body which was most fully studied was of the nature of a "stimulin" or "opsonin."

4. Antitoxic serum obtained by inoculation of culture filtrates does not stimulate phagocytosis of whole culture.

5. Inoculation of *washed* cultures does not evoke the elaboration of so active an "opsonin" (for *whole* cultures) as does inoculation of *whole* cultures.

6. *Washed* culture and *whole* culture appear to be equally active in evoking agglutinin production.

The following deductions therefore seem permissible: that in *whole* cultures of *B. tetani* three antigens, at least, appear to exist.

(a) The *bacillary* substance itself—the inoculation of this leads to the development of agglutinins which are specific to the Types.

(b) An *antigen*, which is anti-phagocytic, is present in young unfiltered cultures; but if present at all, is only found in small quantity in filtrates. The presence of this antigen in an *inoculum* evokes the development of "opsonins" which are specific to the Types.

(c) The *spasm-producing toxin*—an antigen which is filtrable and which, in laboratory animals at least, does not appear to be specific to the Types.

These findings suggest, that improvement might be looked for in serum prophylaxis and serum therapeutics from the employment of sera possessing anti-bacterial as well as antitoxic properties.

The demonstration of specific opsonic activity further suggests, that the typing of the bacilli may be of some import in the pathology of the disease.

III. In Section V the following subjects are considered:

(i) The mechanism of infection in tetanus.

(ii) The influence of antitoxic serum on infection with *B. tetani*, as contrasted with its influence upon intoxication with the products of that organism.

(iii) The relative value of *antitoxic* and *anti-bacterial* sera for prophylactic use.

(iv) The demonstration of "*whole animal*" immunity specific to the Types. This is the natural corollary of the experiments undertaken *in vitro* for the demonstration of *anti-bacterial* bodies specific to the Types. From the results obtained the following conclusions may be drawn:

1. That the *spasm-producing* toxin of *B. tetani*, when employed in sub-lethal doses, does not produce sufficient local devitalisation of tissue to permit of the growth of *B. tetani* when inoculated along with it.

2. That the toxin of *B. Welchii*, and to a less extent that of *Vibrio septique*, when used in sub-lethal doses, do produce sufficient devitalisation of tissue to allow of the development of tetanus infection. Antitoxins to the products of these organisms protect animals against infection with *B. tetani* when such products are used as tissue debilitants.

3. The protection afforded by tetanus antitoxin can only be partial, for, if the degree of tissue devitalisation be great, antitoxin used prophylactically fails to prevent the occurrence of tetanus.

4. The nature of the substance used for producing devitalisation of tissue exerts a profound influence upon the development of tetanus spores in the tissues. In guinea-pigs, saponin produces a lesion which always results in the development of spores of *B. tetani* inoculated along with it. The same reagent fails to initiate tetanus infection in the mouse. Trimethylamine, although it may cause the production of a large slough in mice, only infrequently causes the development of spores of *B. tetani* inoculated along with it. Calcium chloride of such concentration that it produces no obvious local lesion will almost invariably cause tetanus spores to develop in these animals.

5. Just as the products of *B. Welchii* and *Vibrio septique* induce tetanus infection, so certain other relationships, the nature of which is at present not determined, appear to depress the infectivity, or toxogenicity, of certain strains of *B. tetani*.

6. The experiments described in this section suggest, but do not prove, that while *monovalent antitoxic* sera exert no specific neutralising influence on the *spasm-producing* toxins of any one Type, they may nevertheless exert a specific *anti-infective* influence. This *anti-infective* influence is *quantitative*, but not *qualitative* in character.

7. The results so far obtained in the investigation of the relative value of *antitoxic* and *anti-bacterial* sera are equivocal. They suggest, however, that this is worthy of more extended investigation. This cannot be done until larger quantities of specific sera are available.

IV. In Section VI are discussed the results obtained in investigating the influence which various dressings exert upon the anaërobic flora of wounds. From this inquiry it is seen, that the dressings, which we were in a position to investigate, exert but little influence upon the anaërobic present in the wounds.

One important fact emerged from the investigation, namely, that excision of the wound area, irrespective of the dressings employed in treatment, exerts a beneficent influence. Anaërobe infection is relatively less when this procedure is employed and convalescence is established at an earlier date than when other methods have been used.

In connection with this work, experiments were undertaken to determine the antiseptic value of certain of the aniline dyes. It is found that the antiseptic activity of these may be greatly reduced in presence of fresh tissue, although their activity may not be reduced in presence of serum.

Tetanus bacilli may be found in wounds at any time during the process of healing. In one instance *B. tetani* was recovered from a wound 882 days after the infliction of the injury. It is sometimes difficult to demonstrate the presence of *B. tetani* in such circumstances, and several swabs may have to be taken before their presence is appreciated by the observer.

V. Examination of the blood of patients suffering from tetanus showed that agglutination could not be employed as an aid to the diagnosis of the disease—Section VII.

VI. In Section VIII is discussed the possible relationship which might exist between the serological Type of a tetanus bacillus, and the haemagglutinating Type of the individual from whom it was isolated. No relationship could be shown to exist between the two.

VII. In Section IX are discussed two interesting cases of abdominal tetanus.

VIII. In Section X are discussed the results obtained in making an examination of human faeces with a view to demonstrating the prevalence of the various Types of *B. tetani* in the human intestine. Unfortunately, owing to the numerous other and more pressing problems under investigation, this work could not be pursued with the vigour necessary to obtain adequate information which would permit of definite conclusions being drawn.

In conclusion we wish to record our thanks to our friends and colleagues who have assisted in the prosecution of this work by their advice and kindly criticism. To Major-General Sir David Bruce, K.C.B., F.R.S., A.M.S., we are especially indebted for inviting us to undertake the investigations, to Lt.-Colonel M. H. Gordon, C.M.G., R.A.M.C., for freely offered advice and encouragement.

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# THE LABORATORY DIAGNOSIS OF TYPHUS FEVER.

## FURTHER OBSERVATIONS ON THE VALUE AND ON THE SIGNIFICANCE OF THE WEIL-FELIX REACTION.

BY N. HAMILTON FAIRLEY, O.B.E., M.D., MAJOR, R.A.M.C.

*Pathologist, 14th Australian General Hospital.*

*Lecturer and Examiner in Parasitology, Egyptian University.*

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FOLLOWING the capture of Jerusalem and in the early months of 1918 Capt. C. M. Craig, R.A.M.C., obtained a culture of a proteus-like bacillus from the civil Jewish bacteriologist of that city. He was informed that this organism was being extensively used by German and Austrian bacteriologists on the Eastern front for the diagnosis of Typhus Fever. After making some preliminary observations on its agglutination by the sera of typhus patients he forwarded the culture on to this laboratory for further investigation, the preliminary account of which was published by Craig and Fairley (21 Sept., 1918, *Lancet*, pp. 385-6).



In the following pages I propose to put on record a *further series* of personal observations fully supporting the conclusions reached in the above-mentioned report.

### 1. CONCERNING CERTAIN ETIOLOGICAL ASPECTS OF TYPHUS FEVER.

Clinically, Typhus Fever must be regarded as a septicaemia. The typical course of the disease, the temperature chart, the dark macular or petechial rash, the markedly toxic condition of the patient all combine to impress this conception upon even the casual observer. Furthermore, as Nicolle first demonstrated, the virus of Typhus is present in the peripheral blood during the pyrexial period, for it may then be transmitted directly to certain species of monkeys and to guinea-pigs by subcutaneous injection of whole blood. Under natural conditions, however, the virus is transmitted from man to man by the louse (*Pediculus humanus*)<sup>1</sup>. Ricketts and Wilder maintain that blood is not infectious if passed through a Berkefeld filter, but Nicolle reports that the virus is filterable. Many organisms have been suggested as the causative agent in the production of this disease, especially bacillary, diplobacillary, and diplococcal forms. As recently as 1917 Futuki has described a spirochaete resembling *T. pallidum* as occurring in the kidney and supra-renal glands of patients dying of Typhus. The same organism was found in the kidney of a monkey inoculated with Typhus Fever.

The organism having most claim to pathogenicity appears to be the *B. typhi-exanthematici* isolated by Plotz<sup>2</sup> from the blood of typhus patients in 1915. It is a small pleomorphic Gram positive non-motile bacillus growing anaerobically in ascitic fluid sterile tissue media. The sera of convalescents from typhus, according to Rabinowitch, show both positive complement fixation and agglutination reactions for this organism when used as antigen. Olitsky<sup>3</sup>, in his recent immunological studies on this bacillus, has confirmed these serological reactions and has shown the presence in typhus serum of the following specific antibodies, *i.e.* agglutinins, precipitins, immune opsonins, anaphylactic substances and complement-fixing bodies. Numerous other organisms have been described by various investigators until the literature on the etiological aspects of Typhus Fever has become as obscure as it is voluminous. Furthermore despite all past observations on the subject no laboratory test of diagnostic value had been evolved. The recent claims, therefore, of Weil and Felix<sup>4</sup> regarding the diagnostic reliability of their agglutination reaction have become a matter of importance to the clinician and the pathologist alike.

<sup>1</sup> A full account will be found in Nuttall (1917), "The part played by *Pediculus humanus* in the causation of disease." *Parasitology*, vol. x, pp. 44-57, 74-75.

<sup>2</sup> Plotz, Olitsky and Baehr (1915), *Journ. Infect. Dis.* xvii. 1-68.

<sup>3</sup> Olitsky (1917), *Journ. Immunology*, ii. 363.

<sup>4</sup> Weil and Felix (1917), *Wien. klin. Wochenschr.* xxx. 393-9; Viteček, *Ibid.* 967-972.

## THE WEIL-FELIX REACTION.

In 1915 Weil and Felix, while investigating a group of cases thought to be enterica but giving negative Widal reactions, isolated an organism from the urine which was agglutinated by the patient's serum in a dilution of 1 in 200. The serum of nine other cases, all of which proved to be Typhus Fever, likewise agglutinated this organism which was identified as belonging to the proteus group. This organism was named the X 2. Later the same observers isolated from the urine a second strain of a similar bacillus known as the X 19, which differed from X 2 in being agglutinated in a very much higher titre by the sera of typhus patients (1 in 2000). A number of other German and Austrian workers<sup>1</sup> have reported favourably on the reaction during the past year.

## 2. CHARACTERISTICS OF ORGANISMS USED IN THE INVESTIGATION.

The organism utilised in the following investigation proved to be a Gram negative slightly motile short bacillus. It grows anaerobically on all ordinary media such as agar and MacConkey's media, and liquefies gelatin. It produces acid and gas in glucose, saccharose, maltose and mannite, but does not ferment dulcitol or lactose. It produces indol freely and acid without clot formation in milk. Subcutaneous inoculation with 2 c.c. of a 24 hours' broth culture proved non-lethal to guinea-pigs.

## 3. AGGLUTINATION PHENOMENA IN TYPHUS FEVER.

Every case in the following series was a typical clinical case of Typhus Fever. In seven cases complications existed at the time the blood was collected for examination. In three cases broncho-pneumonia was present; in another case there was a pelvic cellulitis secondary to perforation of an ulcer of the rectum; in three other cases parotitis had supervened.

The blood picture was investigated in twelve uncomplicated cases. Absence of leucocytosis, or a definite leucopaenia, was the rule during the first week, but this was generally replaced by a moderate grade of leucocytosis during the second week (10,500 to 14,000 per cmm.). The differential count in the second week showed an absolute and relative increase in the polymorphonuclear neutrophile elements and a decrease in the eosinophile cells.

The agglutination was carried out in all cases on a Garrow's agglutino-meter<sup>2</sup>, the results were read after five minutes rocking. Controls were used in every instance. In a number of cases concomitant observations were made by using the macroscopic method of tube agglutination and incubating at 37° C. for four hours.

With the prevailing temperature conditions of Egypt I have always found the Garrow's agglutinometer a most reliable instrument. Economy of material,

<sup>1</sup> Sterling and Sterling, *Wien. klin. Wochenschr.* xxx. 972-4; Ballner and Finger, *Ibid.* 966-7; Dadez and Kvahelsha (1917), *München. med. Wochenschr.* LXIV. 1379-1381.

<sup>2</sup> Garrow (1917). *Lancet*, i. 262.

the rapidity with which results can be read, and the elimination of the feeble agglutinating power of certain sera upon this organism, are the great advantages of this method.

Using this instrument I regard an agglutination of 1/40 as diagnostic of Typhus Fever. Up to the 6th day of the disease an agglutination in a dilution of 1 in 20 should be regarded as sufficient evidence on which to isolate a case.

### *Analysis of Cases.*

(a) Thirty-five cases were examined during the pyrexial period. The titres of the agglutinations were as follows:

Day	Cases	Titre
5th	1	1/40
6th	2	1/80, 1/320
8th	3	1/80, 1/80, 1/1280
9th	3	1/20, 1/40, 1/640
10th	9	1/80, 1/160, 1/160, 1/320, 1/640, 1/640, 1/1280, 1/1280
11th	2	1/40, 1/1280
12th	5	1/20, 1/80, 1/80, 1/640, 1/1280
13th	5	1/640, 1/640, 1/1280, 1/1280, 1/1280

In five febrile cases the exact day of the illness was not known. The titres of the agglutinations obtained were 1/40, 1/320, 1/320, 1/1280, 1/2560.

(b) The sera of twenty-five convalescent cases gave positive reactions as follows:

Day of convalescence	Cases	Titre
1st	2	1/160, 1/1280
2nd	2	1/20, 1/1280
3rd	2	1/40, 1/160
4th	3	1/160, 1/160, 1/640
5th	2	1/40, 1/160
6th	4	1/640, 1/640, 1/1280, 1/2560
7th	1	1/1280
8th	1	1/640
9th	2	1/160, 1/640
10th	1	1/160
12th	1	1/320
14th	1	1/1280
15th	1	1/640
16th	1	1/80
22nd	1	1/320

Progressive agglutination readings were made in four cases as follows:

Case 1.	3rd day. negative	1/20	2 days after crisis	1/20 positive
	6th "	" 1/20	6 "	" " 1/20 "
	8th "	" 1/20	11 "	" " 1/80 "

This case constituted the mildest clinical type of Typhus Fever of the series.

Case 2.	5th day, negative 1/20	9th day, positive 1/160
	7th " " 1/40	11th " " 1/1280

This patient died of an overwhelming toxæmia on the 12th day of illness.

Case 3.	6th day, positive 1/320
	8th " " 1/640
	10th " " 1/640
	12 days after crisis positive 1/160

This patient on the 6th day of illness developed broncho-pneumonia.

Case 4.	6th day, positive 1/80
	8th " " 1/80
	13th " " 1/640

This patient recovered.

(c) Time incidence in the appearance of agglutinin:

In the series of cases under review the observations made during the first week of the disease are very limited, but in the preliminary report of Capt. C. M. Craig and the writer, the agglutination reactions in twenty-five cases between the 4th and 7th day of the disease were recorded.

These results were as follows:

Day	Cases	Titre
4th	6	1/10, 1/10, 1/40, 1/50, 1/100, 1/1000
5th	10	1/10, 1/10, 1/10, 1/40, 1/50, 1/50, 1/50, 1/50, 1/80, 1/160
6th	6	1/50, 1/50, 1/50, 1/80, 1/100, 1/320
7th	3	1/10, 1/20, 1/320

During the above-mentioned period, though agglutinin was definitely present, its titre was not nearly as high as in the subsequent course of the disease. In the 2nd and 3rd weeks of the disease (*i.e.* in the 2nd week of the febrile period and in the first week of convalescence) the maximum height of the agglutinin curve is attained, as may be ascertained by a perusal of the preceding tables.

A certain proportion of the cases do not develop agglutinin during the course of the fever. In the present series 5 out of 65, or 7·7 % of the cases, failed to agglutinate *B. proteus* in a dilution of 1 in 20 of patient's serum. In four of these cases the agglutinin content was not investigated during convalescence. The remaining case developed agglutinin during the first week of convalescence.

(d) The agglutinin content of the patient's blood and its bearing on the prognosis:

There appears to be no direct relationship between the amount of agglutinin present in the circulating blood and the clinical aspect of the case. In the present series the mildest case had no agglutinin for *B. proteus* until the 2nd day of convalescence, whereas some of the most fatal cases had well marked agglutination established as early as the 5th day of fever. Other cases dying later in the disease (11th to 13th day) only developed agglutinin twenty-four hours before death.

(e) Control reactions:

The sera of 120 known negative cases have been examined for agglutination against this proteus-like organism.

Of the protozoal diseases, twenty-five cases of syphilis, thirty cases of relapsing fever, and thirty cases of malaria (twenty-five cases of sub-tertian infection, four cases of benign tertian, and one case of quartan) were examined with negative results.

The bacterial infections which included cases of influenza, pneumonia, undulant fever, and enterica group yielded negative results with two exceptions.

In these two cases (with Garrow's agglutinator) agglutinations in a titre of 1 in 10 were obtained but the reaction failed with higher dilutions.

Recently Captain C. M. Craig, R.A.M.C., informed me he has observed an agglutination of 1/1000 with *B. proteus* in a definite case of typhoid fever, but he was unable to exclude a previous Typhus infection.

In a group of seven cases of Typhus which were tested against stock culture of *B. typhosus* and *B. paratyphosus* A and B, during the pyrexial period, six yielded negative results. In the other case which had been inoculated with T.A.B. nine months previously, *B. paratyphosus* A and B yielded a positive agglutination with the patient's serum in a dilution of 1/40, while *B. typhosus* yielded agglutination in a dilution of 1/1280. At autopsy infection with *B. typhosus* was definitely excluded in this case.

The sera of convalescent Typhus patients tested against stock culture of *B. shiga*, *B. flexner*, Y, *M. melitensis*, *cholera vibrio*, and *B. coli* all yielded negative results. Similarly *B. proteus* failed to be agglutinated by standard immune sera of *B. shiga*, *B. flexner*, *B. typhosus*, *B. paratyphosus* A and B, and of the cholera serum prepared by the Lister Institute.

#### 4. COMPLEMENT-FIXATION REACTION.

Technique employed; preparation of reagents and of antigen from *B. proteus*.

As the sera of typhus patients agglutinated, in a high titre, a saline suspension of this proteus-like organism, an investigation was carried out by means of the complement-fixation method (Bordet and Gengou, 1901, *Ann. Inst. Pasteur*, xv.):

Complement-fixation reactions are dependent on the fact that when antigen, inoculated serum (? immune body) and complement are mixed together immune body firmly combines with antigen and complement in such a manner that complement can no longer be found free in the mixture. If such a mixture is allowed to stand at a suitable temperature, *i.e.* 37° C., for one hour or more, and to it is added a suspension of red blood corpuscles sensitized with a suitable quantity of haemolytic serum, no haemolysis will take place since there is no free or available complement. This constitutes a positive reaction and proves the presence of specific immune body in the inactivated serum.

If the complement is not fixed then haemolysis ensues; this constitutes a negative reaction and demonstrates the absence of specific immune body in the serum under investigation.

The antigens used were in fresh saline (0.85 % NaCl and 0.5 % phenol) suspension prepared from a twenty-four hours' growth of this organism on agar slopes.

The technique employed was similar to that used in the ordinary *quantitative* Wassermann reaction. Three, six, nine and sometimes twelve minimum haemolytic doses of complement were used in the test.

In the first stage of the reaction, quantities of antigen, immune serum and complement were mixed together for one hour at 37° C. Subsequently sensitized sheep's corpuscles were added and final readings were made after another hour's incubation at 37° C.

The results were recorded as follows:

1.	P + + + +	fixation of 12 M. H. doses of complement		
2.	P + + +	"	9	" "
3.	P + +	"	6	" "
4.	P +	"	3	" "

*Antigen.* The antigens employed in the present investigation were prepared by two methods:

*Antigen A.* This antigen consisted of a fresh saline (*v. supra*) suspension of a twenty-four hours' growth of *B. proteus* on agar slants (this antigen was the one used in fifty-eight cases of Typhus Fever, and in animal and human inoculation experiments).

*Antigen B.* In this method the (fresh) saline suspensions prepared from growths of *B. proteus* on agar slopes (twenty-four hours old), were heated to 56° C. for one hour and then carefully centrifuged. The supernatant suspension was utilised as antigen. (This was used to investigate nine cases of Typical Typhus.) In standardising the antigen it was found advisable never to use more than one-third the anti-complementary dose.

*Haemolytic serum* was obtained from rabbits by injecting intraperitoneally and intravenously sheep's corpuscles in progressively increasing doses. The serum used in these tests was one of high titre (1/4000). To sensitize the sheep's corpuscles four minimum haemolytic doses ofamboceptor or haemolytic serum were used. The M.H.D. of the amboceptor was taken to be that amount of haemolytic serum just sufficient to produce in one hour at 37° C. complete lysis in one volume of a 3 % suspension of sheep's corpuscles with four or five M.H.D.'s of complement.

*Sheep's corpuscles.* Equal quantities of sheep's blood were mixed with 2 % sodium citrate in physiological saline. Requisite amounts of this mixture received three washings with nine times the volume of physiological saline, and were finally made up to the equivalent of a 3 % suspension of sheep's corpuscles in the same solution.

## *Laboratory Diagnosis of Typhus*

*Sensitization of corpuscles.* After four M.H.D.'s of amboceptor had been added, the suspension of corpuscles was placed in the incubator at 37° C. for thirty minutes, and after sensitization kept in the ice-chest till required.

*Patient's serum.* Blood was usually obtained on the day preceding the test, and kept in the ice-chest till required. The serum was diluted with four times its volume of physiological saline, and heated to 55·5° C. for twenty minutes. Heating in this manner destroys complement and inhibits the anti-complementary properties of certain sera.

*Complement.* The complement used was obtained from well-nourished guinea-pigs, and collected under sterile conditions. The M.H.D. of complement was always obtained by preliminary titration. The reagents were measured out by means of Donald's dropping pipettes.

The arrangement of the systems for the final tests was as follows:

Row No. 1.	Antigen	1 vol.	} + 2 vols. saline (0·85 %)
	Patient's serum	1 „	
	Complement (3 M.H.D.'s)	1 „	
Row No. 2.	Antigen	1 „	} + 1 vol. saline
	Patient's serum	1 „	
	Complement (6 M.H.D.'s)	2 vols.	
Row No. 3.	Antigen	1 vol.	}
	Patient's serum	1 „	
	Complement (9 M.H.D.'s)	3 vols.	
Row No. 4.	Patient's serum	1 vol.	} + 3 vols. saline
	Diluted complement (3 M.H.D.'s)	1 „	

Row No. 4 serves as a serum control and any anti-complementary tendency in each serum examined is thereby demonstrated.

Additional controls used in the test were:

- (1) Antigen control, i.e. 1 vol. of antigen and 4 vols. of saline (0·85 %).
- (2) Antigen 1 vol., pooled negative serum 1 vol., 3 vols. of saline (0·85 %).
- (3) Where possible a sure positive serum was included in the series (i.e. a monkey inoculated with *B. proteus*).

### ANALYSIS OF RESULTS.

#### *Complement-fixation reactions in the sera of typhus patients.*

In all fifty-eight sera collected from typhus cases in different stages of the disease were examined. Their agglutination reactions were as follows:

In	1 case	the titre was	1/2560
„	13 cases	„ „ „	1/1280
„	15 „	„ „ „	1/640
„	6 „	„ „ „	1/320
„	9 „	„ „ „	1/160
„	6 „	„ „ „	1/80
„	5 „	„ „ „	1/40
„	3 „	„ „ „	1/20

As far as possible blood was collected from the cases during the second week of fever or in the first week of convalescence (*i.e.* 8th–21st days), during that period when the agglutinin content was at a maximum.

In fifty-five out of fifty-eight of the cases examined the complement-fixation reactions were negative. In one case a P+++ reaction was obtained, in another case there was a similar reading, but the control showed the serum to be anti-complementary. In one other case a P++ reaction was recorded.

#### *Examination of non-typhus sera.*

Eighty-three sera from non-typhus cases were examined. Of these eighty yielded negative results and three yielded pseudo-positive reactions. One case of rheumatic fever gave a P++++ reaction, and two cases of syphilis yielded a P++ reaction. The sera of twelve other cases of syphilis yielding positive Wassermann reactions were negative.

#### *Investigation of the amount of complement fixed by pooled typhus serum in the presence of B. proteus as antigen.*

A pooled serum was prepared from nine cases of Typhus all of which yielded high titre agglutinations. The amount of complement fixed by this serum in the presence of *B. proteus* as antigen was found to be identical with the amount fixed by a pooled serum prepared from eleven non-typhus patients. Less than 1½ M.H.D.'s of complement were fixed in each system.

*Conclusion.* It follows from the above experiments that the formation of agglutinin in the blood of typhus cases for this *particular kind of B. proteus* is not accompanied by the formation of immune body, as indicated by the complement-fixation reaction.

#### EXPERIMENTS ON ANIMALS.

Ten monkeys were experimented on (seven *Cercopithecus* and three *Macacus rhesus*).

Of these ten monkeys four were used as controls throughout the investigation. The six others were injected sub-cutaneously with one dose of from 1 to 2 c.c. of a twenty-four hours' broth culture of *B. proteus*. In only one of these monkeys was a second injection given and this was to a monkey labelled "Z" at an interval of ten days following the first injection.

In every case, prior to inoculation, the serum of the monkey was investigated for complement fixation and agglutination reactions against this organism. All had negative complement fixation, and all yielded negative agglutination reactions in dilution of one in ten with one exception only. In this case (monkey "W") a positive agglutination of monkey's serum in a titre of one in ten was obtained but the reaction vanished in a higher dilution of 1 in 20. None of the inoculated monkeys showed obvious signs of sickness.

A perusal of Table I will show the serological reactions of monkeys inoculated sub-cutaneously with broth cultures of this organism.



An analysis of the protocol will also show that in all cases a positive complement-fixation reaction was obtained. In five out of the six cases this was present within twelve days of inoculation. In the sixth case (monkey "D") the complement-fixation reaction was negative on the ninth day, though it became positive on the eighteenth day. The agglutination response in this case was not as intense as usual.

In all these cases accompanying the appearance of agglutinin in the blood serum of the inoculated monkeys, immune bodies were produced which had the property of fixing complement in the presence of its specific antigen, namely *B. proteus*.

#### INOCULATION OF MAN WITH *B. proteus*.

In order to observe the serological and clinical effects produced by sub-cutaneous injections of *B. proteus* two of the laboratory staff (R. C. S. and N. H. F.) were inoculated sub-cutaneously with a saline suspension of 3000 million living *B. proteus*. In both cases a systemic reaction followed (generalised aches and pains, headache, fever 100–102° F.); but these symptoms disappeared within forty-eight hours. A local inflammation occurred at the site of inoculation. In one case resolution without suppuration followed, in the other, on the ninth day, the site of inoculation was incised. A small amount of *sterile pus* was present.

Table I.

#### *Serological Reactions in Infected Monkeys.*

Days after inoculation	MONKEY "Z"		MONKEY "W"		MONKEY "X"		MONKEY "V"		MONKEY "D"		MONKEY "A"	
	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation	Agglutination	Complement fixation
4	1/20	—	—	—	—	—	—	—	—	—	—	—
6	1/160	P + + + +	1/320	—	—	—	—	—	—	—	—	—
9	1/640	—	1/2560	P + + + +	1/1280	P + + + +	—	—	1/150	Neg	—	—
12	1/1280	P + + + +	—	—	—	—	1/640	P + + + +	—	—	1/160	P + +
18	1/1280	P + + + +	—	—	1/640	—	—	—	1/320	P + + + +	—	—
25	—	—	1/320	P + + + +	1/320	P + + + +	—	—	1/320	P + + + +	—	—
35	1/640	P + + + +	—	—	—	—	—	—	—	—	—	—
53	1/40	Neg.	1/160	P + +	1/160	P + +	—	—	1/20	Neg	—	—

Table II.

#### *Serological Reactions in Man inoculated with living B. proteus.*

Days after inoculation	Case 1		Case 2	
	Agglutination	Complement fixation	Agglutination	Complement fixation
Before	Neg.	Neg.	Neg.	Neg.
6th day	1/360	P + +	1/160	P +
13th "	1/160	P +	1/640	P + +
18th "	1/80	Neg.	1/640	P + +

The result of the serological reactions observed will be found in Table II. In both cases agglutinin and complement fixing bodies appeared in the circulating blood a few days after inoculation.

##### 5. SIGNIFICANCE AND EXPLANATION OF THE WEIL-FELIX REACTION.

Naturally the significance of the Weil-Felix reaction has caused much discussion. The general consensus of opinion as quoted in a leading article in the *British Medical Journal* (12. I. 1918), including that of Weil and Felix themselves, is that *B. proteus* is not the real cause of Typhus, but a specific secondary invader of the body which accompanies the unknown virus of the disease.

That *B. proteus* is not the causative agent in Typhus is indicated by the facts that (1) it cannot be isolated from blood cultures made during the pyrexial period; (2) it is non-pathogenic to man and monkeys when inoculated sub-cutaneously.

The secondary invasion hypothesis, while superficially plausible and while accounting for the presence of high titre agglutinins in the blood serum of typhus cases, is incompatible with the following facts:

(1) Systematic cultural investigations of the blood of typhus cases over different periods of the disease and also of the urine, save in very rare cases<sup>1</sup>, yield negative results, even though *B. proteus* grows aerobically and very readily on all the ordinary laboratory media; furthermore it must be remembered that in any case systematic cultural examinations of the urine of any large series of cases will reveal an occasional *B. proteus* infection, hence the cultural findings of Weil and Felix are by no means conclusive.

(2) Serological investigations of typhus sera, while showing the presence of agglutinin for *B. proteus*, have failed to demonstrate the presence of immune body; whereas sub-cutaneous injections into monkeys and man have been followed by the production of agglutinin and complement fixing antibody.

My observations here are entirely in *disagreement* with those of Wagner (1917, *München. med. Wochenschr.* p. 792), who claims to have obtained positive results with the complement-fixation test using *B. proteus* as antigen in five out of six cases of Typhus so investigated.

*Per contra my results showed:*

(1) No increased tendency for fixation of complement by a pooled typhus serum in the presence of *B. proteus* antigen over that quantity fixed by a pooled negative serum under similar conditions.

(2) Fifty-five out of fifty-eight cases of definite Typhus Fever yielded negative complement-fixation reactions.

(3) Using an identical technique, monkeys and man after sub-cutaneous inoculations invariably yielded positive complement-fixation reactions.

<sup>1</sup> Craig and Fairley, *loc. cit.*

In consequence of these findings, and in contra-distinction to the generally accepted view, I hold that the only satisfactory explanation of the Weil-Felix agglutination reaction is to regard the phenomenon as due to a secondary or heterologous agglutinin (*Nebenagglutinin*).

It would be a matter of considerable interest to investigate the residual agglutinin in typhus sera after saturation with suspensions of *B. typhi-exanthematici* Plotz and *B. proteus* Weil and Felix. Additional data might thereby be obtained concerning the etiological significance of both micro-organisms.

Posselt and Sagasser (1903, *Wien. klin. Wochenschr.* No. 24) showed that in immunisation there is not only an increase in the amount of agglutinin for the organism injected but also of secondary agglutinins (*Nebenagglutinine*) which act on other organisms. Thus they found that while the serum of a guinea-pig immunised against *B. typhosus* contained specific agglutinin for that organism, even in a titre of 1/12,000, it also had developed secondary heterologous agglutinins for *V. cholerae* of a titre of 1 in 4500, and for *B. dysenteriae* of a titre of 1 in 4000.

Later Ballner and Sagasser showed that at times these secondary agglutinins are markedly increased and that inoculation with certain organisms like *B. tetani* and Friedländer's bacillus, while leading to the formation of but few specific agglutinins (*Hauptagglutinine*), produced numerous secondary heterologous agglutinins.

A review of the literature certainly leads to the conviction that the sera of typhus cases must be particularly rich in these secondary agglutinins.

Thus agglutinins in typhus sera have been described by the following observers for the following micro-organisms:

- (1) For *B. typhi-exanthematici* by Plotz (*loc. cit.*).
- (2) For *Bacillus* "U" by Wilson (*Journ. Hygiene*, 1909, ix. 316-337; *ibid.* 1910, x. 155). This organism is a variant strain of *B. coli* and was isolated from the faeces of a case of Typhus during the Belfast epidemic of 1908. Wilson attributes this agglutination to the presence of secondary heterologous agglutinin.
- (3) For *Bacillus typhosus*, Wilson (*loc. cit.*) reports positive agglutination in a titre of 1 in 50 as existing in the sera of eighteen out of thirty-one cases of Typhus, and concluded that the Widal reaction was of no value in distinguishing Typhus Fever from Typhoid.
- (4) Hornicki, in Manchuria, isolated an organism allied to *Bacillus* "U" of Wilson from the urine and faeces of typhus cases and reported positive agglutination reactions with the sera of typhus patients.
- (5) Weil and Felix (*loc. cit.*) have described two strains of *B. proteus* (the X 2 and the X 19) which have already been referred to in detail.

Wherein lies the explanation of this almost promiscuous agglutinating action of typhus serum on a number of biologically distinct species of micro-organisms?

Surely it is impossible for *all* these organisms to be constant secondary invaders in Typhus Fever. Is it not more rational to think that the virus or specific agglutinin of typhus, whilst stimulating homologous receptors or specific agglutinins, also has the property of stimulating other closely related receptors of secondary agglutinins, which agglutinate micro-organisms of different biological strains? Such a hypothesis would afford an explanation not only of the presence of agglutinin for *B. proteus* and the absence of specific immune body for that organism in the sera of typhus cases, but also of the wider agglutinating properties possessed by typhus sera in general.

Furthermore such a conclusion need not detract from the value of the Weil-Felix reaction as a laboratory aid to the diagnosis of Typhus Fever.

The fact that the Wassermann reaction is not a specific test for syphilis, in the strict immunological sense, has not diminished its practical application as a diagnostic test for that disease.

In similar fashion the appearance in the sera of typhus patients of the agglutinin for an organism, which has no apparent relationship to the disease, need not bias the student against the great diagnostic value of this reaction.

#### CONCLUSIONS.

(1) The Weil-Felix agglutination reaction has again proved, in a further series of cases, to be a very reliable laboratory aid to the diagnosis of Typhus Fever.

(2) Frequently the reaction becomes definitely established during the first week of the disease. The maximum agglutination readings are obtained during the second week of fever and during the first week of convalescence (*i.e.* 8th to 21st days).

(3) Of sixty-five cases of definite Typhus Fever sixty-three or 94 % yielded positive agglutination reactions.

(4) Of 120 non-typhus sera no case yielded positive agglutination in a dilution of 1 in 20, utilising Garrow's method of agglutination. In two cases an agglutination in a dilution of 1 in 10 was obtained. On the Garrow's agglutinator a positive agglutination of patient's serum in a titre of 1 in 40 may be regarded as diagnostic of Typhus Fever. A positive reaction in a dilution of 1 in 20 of patient's serum may be regarded as sufficient evidence on which to isolate a case during the first week of illness.

(5) The appearance of the agglutinin in the sera of typhus cases for the *B. proteus* utilised in the present investigation is not accompanied by the formation of specific immune body. On the other hand living cultures inoculated sub-cutaneously into monkeys and man are followed, not only by the appearance of agglutinin, but also by the production of immune body as revealed by the complement-fixation test, utilising *B. proteus* as antigen.

(6) The hypothesis that *B. proteus* is a constant secondary invader accompanying the unknown virus of Typhus Fever lacks confirmation and is

incompatible with certain ascertained facts. The Weil-Felix reaction is dependent on the presence in typhus sera of a secondary non-specific agglutinin which has the property of agglutinating this *proteus*-like organism.

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For permission to publish this additional report I am indebted to my C.O., Colonel Walter Summons, A.A.M.C.

# FURTHER EXPERIMENTS IN THE ETIOLOGY OF DENGUE FEVER.

BY

J. BURTON CLELAND, M.D., CH.M. (SYD.), Principal Microbiologist, Microbiological Laboratory, Department of Public Health, Sydney,

AND

BURTON BRADLEY, M.B., CH.M. (SYD.), D.P.H. (LOND.), Director Laboratory of Microbiology and Pathology, Brisbane; late Assistant Microbiologist, Department of Public Health, Sydney, and Hon. Pathologist, Royal North Shore Hospital of Sydney.

(Clinical notes by W. MACDONALD, M.B., CH.M., Medical Superintendent, Liverpool State Hospital.)

(With 9 charts.)

(From the Laboratories of the Department of Public Health and of the Royal North Shore Hospital of Sydney.)

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## INTRODUCTION.

IN a previous communication(1), after reviewing the literature of the subject, and especially the claims as to the transmission of dengue by *Culex fatigans*, we were able to show conclusively that in Australia the Yellow Fever mosquito, *Stegomyia fasciata*, is capable of transmitting the virus of dengue. Whether or not other mosquitoes can also play a part we cannot say, although epidemiologically we think this unlikely. We also showed that the incubation period was certainly over five days, and usually between five and a half and nine and a half days, in our mosquito cases. We showed also that the virus could be transmitted by the subcutaneous inoculation of blood.

In a further communication(2) we showed *inter alia*:

1. That the virus of dengue may be present in the whole blood, the serum, the washed corpuscles or the citrated plasma.
2. That the virus was present in the blood on the second and third days of the disease (24 to 48 hours after onset), and possibly as late as the eighth day (about 168 hours after onset). Some evidence was adduced to show that the virus was not present in the blood on the fourteenth day of the disease.
3. That our filtration experiments were not conclusive. There was one apparently successful case out of five experiments. In some of these, for other reasons, the result might have been expected to be negative.
4. That the incubation period varied from six to nine days.
5. That immunity to inoculation was present twenty-four days after recovery from dengue.
6. That blood containing the virus may retain its infectivity in a cool place outside the body for at least seven days.
7. That the infection of dengue could be conveyed to at least four "generations" of artificial (injected) cases.

The present series of experiments is the outcome of a project which to a great extent has not materialised. We had intended to establish a chain of artificial dengue cases in volunteers, to keep the virus alive in the series by successive injections, and with this virus to perform as it were a series of side-chain experiments on other volunteers to obtain certain special aspects of the problem. We had made arrangements to breed out mosquitoes and had devised experiments to confirm our previous findings and to determine several important points as to the nature of the transportation of the virus by these insects. We had also planned to conduct experiments as to the nature of the virus in man, and especially as regards its filtrability, its resistance to outside influences, its persistence in the blood and its distribution in the elements of the blood.

Certain facts combined caused us to greatly curtail our scheme and eventually the virus-chain ran out and further experiments were abandoned. This was chiefly due to the unexpected difficulty of obtaining volunteers, even with a considerable monetary inducement. The mosquitoes which we were able to breed for some reason did not bite well and died off rapidly.

This should be borne in mind in considering the experiments herein described. At first sight, in several instances, we seem to have neglected opportunities for experiments, whereas in reality we had no volunteer available at the time.

Again, in two experiments, the finding of a positive complement fixation reaction for syphilis in the volunteer prevented further utilisation of the virus in his blood.

In spite of these difficulties we have been able to obtain further information on certain points as to the dengue virus.

The present experiments show in brief:

1. That it is possible by subcutaneous injection of blood, or derivatives of blood, from a dengue patient to transmit the disease to a healthy non-immune, and from this case in

a similar way to derive a series of experimental cases. In the present communication the original virus obtained from a natural case has been passed successively through four "generations" of artificial cases, with a doubtful positive case in the fifth "generation."

2. That the virus under certain conditions may be passed through a Pasteur-Chamberland F. filter which at the same time is keeping back ordinary organisms (staphylococci, colon bacilli).

3. That the virus does not appear to be specially contained in any one element of the blood. Carefully washed corpuscles, citrated plasma, and serum free from corpuscles all contain the virus. It is not absolutely certain, however, in the case of serum that the virus may not obtain access to this by the breaking up of corpuscles.

4. That the virus is resistant to conditions outside the body for several days (99 hours).

5. That in the blood of the dengue patient the virus was present in one case 18 hours after the onset, in one case 67 hours and in another as late as 90 hours, whilst several cases show it to have been present at 57 hours and less after the onset. It was not found in a case 130 hours after the onset.

6. That the incubation period, while variable and not shorter than  $4\frac{1}{2}$  days, is usually from 6 to 8 days, but may be as long as 15 days.

7. That immunity may be present to injection 229 days after the onset of a previous attack of dengue.

8. That, in one experiment made, no evidence could be found that *Culex fatigans* can transmit the virus of dengue.

9. That, in one experiment made, no evidence was found that the blood of a guinea-pig contained the virus seven days after its inoculation.

10. That no result followed inoculation of guinea-pigs or rabbits, and no pathological lesions were found in inoculated guinea-pigs and no spirochaetes were found in their organs by Ievaditi's method.

#### PART I. INJECTION EXPERIMENTS.

Reviewing the cases of the present series, certain peculiarities are shown which may be briefly summarised.

The type of the disease, as in fact is the case in the natural illness, varied very greatly. Certain of our cases were typical of dengue, whilst others were only recognisable as such by the temperature and pulse variation and the nature of the surrounding circumstances. A striking phenomenon is the mildness of the symptoms in the aged volunteers from the Asylum, in contrast to the general severity of the cases in the younger and more active volunteers from our staff and from that of the Royal North Shore Hospital. As will be mentioned later, mild symptoms were often associated with a very definite pyrexia.

Cases 2, 4, 15 and 17 were all classifiable, as regards symptoms, as of "typical rather severe" type. That is to say, they all had marked general body and eye pains and headache with considerable prostration, nausea and malaise, and all looked ill. The rashes in these cases were a prominent feature, and complete recovery was slow, probably in all. Now it is extremely interesting to note that whereas Cases 2 and 4 are at the beginning of our series, Cases 15 and 17 are both fourth generation cases and are separated from the two severe early cases by comparatively mild cases, Case 15 being derived



from Case 10, and Case 17 from Case 11—both Case 10 and 11 being descendants of Case 4. In Case 10, for instance, the volunteer only complained of symptoms for one day and the rash was extremely faint, whereas the descendant Case 15 was feeling very sick for three days, and had a most distinct rash and typical symptoms.

Case 17 was of considerable severity with a distinctly typical rash, whereas Case 11, although showing definite though slight symptoms and a definite slight rash, was altogether much milder than Case 17.

The febrile reaction in most cases was distinct and constituted in some the chief evidence as to the presence of the infection. Some of the cases were not charted in detail but in most cases we have a detailed record of the temperature and pulse from the date when the virus was administered to some time after the end of the febrile phase. The course adopted in this series was to take the temperature twice daily, usually for a week, and if no symptoms or pyrexia declared itself in this period to take the chart four-hourly for the remaining period. The total period of observation was usually between three and four weeks.

A review of Table I and of the charts will indicate, better than a description, the type, duration, and severity of the febrile reaction. It will be seen that the febrile period ranges from somewhat over three days to seven days. In most cases it is easy enough to determine the point of onset of the pyrexia, as also its termination, but occasionally there may be a slight elevation of temperature to about 99° F. for a day or more before or after the termination of the definite fever.

*The degree of fever:* A review of the maximal points in nine of the positive charted cases shows the lowest temperature to be 100.8° F. and the highest 104.6° F. 102° F. would be about the average maximum temperature and in four cases it lies between 102–103° F. The fever seems hardly at all correlated with the symptoms—in some of our cases, especially in Case 14, the want of correlation is especially distinct. This particular case had virtually no symptoms with a temperature of nearly 105° F., which remained high for nearly two days, then fell and rose again to nearly the same level with another short interruption, and then fell abruptly.

*Type of fever:* Although several of the charts are definitely saddle-back in type, and others show a more irregular diphasic variation, others again show an irregular temperature curve. We do not think the type of temperature is at all diagnostic of Australian dengue, either in natural or artificial cases. Although certain cases may show typical saddle-back charts, and as may be seen in our previous papers there may be two separate pyrexial phases, yet other typical cases may show a simple monophasic variation or irregular or plateau types of chart.

*The pulse and its relationship to the pyrexia:* As shown in our previous papers and noted by other observers in natural cases, the pulse of dengue has a distinct tendency to be slow relatively to the degree of fever. This may

Table I.

Case No.	Initials	Result	No. of days during which temp. taken	Incub. period in days	Pyrexia			Pulse during Pyrexia.			Remarks
					Type	Highest temp. °F.	Duration in days	Days charted after Pyrexia	Rel.	Abs.	
2	W. G. A., m.	Pos.	15	8	Irreg.	102.8°	6	1	+	...	Rash
3	B. B., m., 34	Neg.	Not taken	...	...	...	...	...	...	...	++
4	H. G., m., 40	Pos.	Not taken	6½	...	About 102°	...	...	...	...	Immune
5	P. N., m., ?	Neg.	33	...	(See history)	...	...	...	...	...	++
6	H. D., m., 45	Pos.	28	15	Irreg. diphasic	100.8°	7	6	+	...	Doubtful case.
7	W. J. I., m., 72	Neg.	21	...	...	...	...	...	...	...	++
8	J. W. M., m., 63	Pos.	23	10	Irreg. diphasic	101°	7½	6	+	-	...
9	J. W., m., 65	Neg.	22	...	...	...	...	...	...	...	...
10	P. M., m., 45	Pos.	23	9	Irreg. diphasic	102.6°	7	7	++	-	Rises to 99° after pyrexia
11	J. D., m., 74	Pos.	21	7	Plateau	102.4°	3 +	11	++	-	Later oscillations to 99°
12	P. B., m., 61	Neg.	23	...	...	...	...	...	...	...	...
13	R. C., m., 58	Pos.	20	8?	Irreg.	100.8°	6?	7	++	+	Temp. 99° one day before onset
14	H. W., m., 70	Pos.	17	5	Typical saddleback	104.6°	7	5	++	-	Whole chart not shown
15	W. M., m., 30	Pos.	12	7	Incomplete chart, prob. irregular	102°	4	1	-	-	++
16	A. A., m., ?	Neg.	21	7	...	...	...	...	...	...	...
17	N. K., f., 28	Pos.	...	?	No chart	100.3°	...	...	...	...	...
18	J. F. H., m., 70	Pos.	19	4½	Irreg.	100.8°	5	9	++	...	...
19	T. B., m., 55	Neg.	23	...	...	...	...	...	...	...	...
20	C. L., m., 70	Pos.?	...	...	...	...	...	...	...	...	...
21	H. McD., m., 42	Neg.	Not charted	...	...	...	...	...	...	...	...

be accompanied by absolute bradycardia (pulses between 50 and 55 are fairly frequent, especially after the pyrexia). Although several of our charts in this series show periods when the pulse is between 50 and 55, definite absolute bradycardia is not a very prominent feature. On the other hand the

relative bradycardia is present in every case of which we have complete charts, and in some of these is very distinct.

*Rashes:* No attempt was made in this series to separate primary and late rashes and detailed descriptions of the rashes are not given. Where it occurred it corresponded to our previous description and was typical of dengue and not readily to be confused with that of any other complaint. In the Asylum cases the rashes were slight, but in the cases among the younger more active volunteers from this staff and elsewhere the rashes were very prominent and characteristic.

Out of eleven cases which were regarded as positive, five showed distinct and typical rashes; one showed a definite, but less distinct, rash, whilst two cases showed only a slight rash which was not prominent enough to be noticed unless specially sought for. The other cases, although they showed an erythematous flushed skin, could not be said to have a rash at any period of their illnesses.

*Symptoms:* These need not be detailed here. Reference to the Appendix will show that they were prominent and typical in the outside cases but mild, sometimes practically absent, in the institution cases. The experience referred to above, in which mild cases intervened in the series between severe cases, teaches us to attach less importance to symptoms in our class of institution volunteers and to rely more on the temperature-pulse variation for determining the positivity of the cases.

#### INFORMATION BEARING ON THE NATURE OF THE VIRUS, ETC., TO BE OBTAINED FROM THE CONSIDERATION OF THE CASES IN THIS SERIES.

Table II indicates the relationship of the human inoculation experiments in this series; it records briefly the nature of the injection, date of injection, date of onset, and the incubation period.

Certain special aspects of the experiments are discussed here (see Appendix, where full experimental and clinical data are recorded).

*Transmissibility of dengue:* As we and others have shown that dengue fever can be successfully transmitted by the blood of a person with the fever to a non-immune and on through several generations, this matter need not be elaborated here. In the present series the virus has been transported through four "generations" of artificial cases. The type and severity of these cases have been discussed above.

*Time during which the blood contains the virus:* Table II shows the duration of the illness in the previous case at the time the material was taken, the period spent by the virus outside the body, and the nature of the material used for injection, correlated with the results of the experiments. This table shows that the virus was present in the blood of the dengue patient as late as 90 hours after the onset in one case; 67 hours in one case; 57 hours in three cases; 47 hours in two cases; 46 hours in one case; 22 hours in two cases; and 18 hours in one case.

Therefore these cases demonstrate that the virus is present in the blood as early as 18 hours after the onset of symptoms and may be present as late as 90 hours after. As we have only one case showing this we cannot be certain that this long period may not be exceptional. However, between 60 and 70 hours after the onset the virus is probably usually present in the blood.

As regards injections followed by negative results, only two of these can be fairly used as indicating an absence of infectivity of the blood. The remaining cases might for other reasons have given a negative result. Case 21, which was negative, injected with the blood of an atypical case taken only a few hours after the onset, will be specially discussed. Cases 12 and 16 were injected with untreated blood. The virus used for the injection of Case 12 was outside the body less than 24 hours and that of Case 16 only a few hours. In Case 12 the blood was taken from Case 4 on the sixth day of the illness (approximately 130 hours after the onset). In Case 16 the blood was derived from Case 10 on the ninth day of the disease (approximately 190 hours after the onset). Both Case 4 and Case 10 were shown to have had the virus in the blood at an earlier stage. From these two cases it may be deduced that the virus was not present in the blood at 130 hours and 190 hours after the onset.

*Summary: The present figures show that the virus may be present in the blood as early as 18 hours and as late as 90 hours after the onset. It was not found in the blood 130 hours and 190 hours after the onset.*

*Resistance of dengue virus outside the body.* The virus seems to resist well for a short period the conditions of a sojourn outside the body. As far as possible when preserving blood for injection we have made use of an ice-chest but in several cases the material was transferred between the laboratories and the hospital with no special precautions, and filtration was performed at room temperature without destroying the virus.

In one instance the untreated blood was preserved, chiefly in the ice chest, for 99 hours before injection and gave rise to a typical case (Case 8). There were several instances where the virus was outside the body about 48 hours, and in one case after 72 hours the virus was possibly still active (doubtful Case 20).

*Summary: The virus outside the body has been found alive, if kept reasonably cool, in several instances up to 48 hours, and in one instance after 99 hours.*

*It is not possible from this series to draw deductions from the negative cases on this question.*

*Filterability of the virus.* In our last communication we reported one apparently positive case and several negative cases after filtration. The single positive case was not fully controlled. In the present series we obtained four negative and three positive results (see Table II). \*

As regards the negative results, Case 19 can be excluded as the blood was taken, from Case 11, 115 hours after the onset, and our experiments suggest that after this period the virus may have disappeared from the blood.

Table II. *Showing sequence of experiments.*

(1) Dr F G. (natural case). Ill, 11-1-17 (evening). Blood taken, 13-1-17 (4 p.m.)	
(2) Dr W G A. Inj. 0.5 c.c. dil. blood, 15-1-17 (4 p.m.). Ill, 23-1-17, 5 p.m. Incubation period, 8 days. Result— <b>Positive</b> .	(3) Dr B. B., m., 34 yrs. Inj. 0.5 c.c. dil. blood, 15-1-17 (4 p.m.). Result— <b>Negative</b> .
	Blood taken, 24-1-17 (11 a.m.)
(4) H. G., m., 40 yrs. Inj. 1 c.c. diluted blood, 24-1-17 (2.30 p.m.). Ill, 31-1-17, 3 a.m. Incubation period, 6½ days. Result— <b>Positive</b> .	Blood taken, 27-1-17 (11 a.m.)
	(5) P. N., m. Inj. 9 c.c. diluted filtered blood, 25-1-17 (7.4.30 p.m.). Result— <b>Negative</b> .
(6) H. D., m., 45 yrs. Inj. 0.5 c.c. blood, 27-1-17 (5.30 p.m.) Ill, 11-2-17. Incubation period, 15 days. Result— <b>Positive</b> .	Blood taken, 5-2-17 (noon?). (12) P. B., m., 61 yrs. Injected 0.3 c.c., 6-2-17 (10 a.m.). Result— <b>Negative</b> .
	Blood taken, 2-2-17 (noon).
(7) W. J. I., m., 72 yrs. Inj. 2 c.c. diluted filtered blood, 1-2-17 (4.30 p.m.). Result— <b>Negative</b> .	

<p>(8) J. W. M., m., 63 yrs. Inj. 0.8 c.c. blood, 6-2-17 (3 p.m.). Ill, 16-2-17 (2 p.m.). Incubation period, 10 days. Result—<b>Positive</b>.</p>	<p>(9) J. W., m., 65 yrs. Inj. 2.8 c.c. blood filtrate, 3-2-17 (4.30 p.m.). Result—<b>Negative</b>.</p>	<p>(10) P. M., m., 45 yrs. Inj. 1.3 c.c. washed corpuscles, 3-2-17 (4.30 p.m.). Ill, 12-2-17 (5 p.m.). Incubation period, 9 days. Result—<b>Positive</b>.</p>	<p>(11) J. D., m., 74 yrs. Inj. 2 c.c. washings, 3-2-17 (4.30 p.m.). Ill, 10-2-17 (4.30 p.m.). Incubation period, 7 days. Result—<b>Positive</b>.</p>
<p>(13) R. C., m., 58 yrs. Inj. 2.3 c.c. plasma filtrate. 15-2-17 (4 p.m.). Ill, 23-2-17 (4 p.m.). Incubation period, 8 days. Result—<b>Positive</b>.</p>	<p>(14) H. W., m., 70 yrs. Inj. 0.3 c.c. blood, 15-2-17 (4 p.m.). Ill, 20-2-17 (3 p.m.). Incubation period, 5 days. Result—<b>Positive</b>.</p>	<p>(15) W. W., m., 25 yrs. Inj. 2 c.c. serum filtrate, 16-2-17 (10 a.m.). Ill, 23-2-17 (on rising). Incubation period, 7 days. Result—<b>Positive</b>.</p>	<p>(16) A. A., m. Inj. 1 c.c. blood, 20-2-17 (5 p.m.). Result—<b>Negative</b>.</p>
<p>(20) C. L., m., 70 yrs. Inj. 1.2 c.c. blood, 27-2-17 (7 p.m.). Incubation period (?). Result—Doubtful.</p>	<p>(17) N. K., f, 28 yrs. Inj. 2 c.c. (?) blood filtrate, 14-2-17 (time not noted). Ill between 21 and 26-2-17. Incubation period, doubtful. Result—<b>Positive</b>.</p>	<p>(18) J. F. H., m., 70 yrs. Inj. 2.6 c.c. washings, 14-2-17 (3 p.m.). Ill between 18 and 19-2-17. Incubation period, about 4½ days. Result—<b>Positive</b>.</p>	<p>(19) T. B., m., 55 yrs. Inj. 2.3 c.c. serum filtrate, 18-2-17 (10 a.m.). Result—<b>Negative</b>.</p>
<p>(21) H. McD., m., 42 yrs. Inj. 2 c.c. blood, 16-3-17 (4.30 p.m.). Result—<b>Negative</b>.</p>	<p>Blood taken, 13-2-17 (3 p.m.).</p>	<p>Blood taken, 15-2-17 (about noon).</p>	<p>Blood taken, 20-2-17 (time not noted).</p>
<p>Blood taken, 14-3-17 (time not noted).</p>	<p>Blood taken, 24-2-17 (time not noted).</p>	<p>Blood taken, 15-2-17 (3 p.m.).</p>	<p>Blood taken, 15-2-17 (about noon).</p>

Table III.

*Showing the data concerning materials used for injection in relation to the results obtained.*

Case	Result	Incubation period in days	Materials injected			Rash	Remarks
			Nature and source	Hours after onset*	Hours outside body†		
(1) Dr F. G., m.	Pos.	Over 5	0.5 c.c. (1 to 2) diluted blood from Case 1	Naturally acquired		- +	
(2) Dr W. G. A., m.	Pos.	8		46	48	- +	
(3) Dr B. B., m., 34 yrs.	Neg.	...	Same as No. 2	46	48	-	Immune
(4) H. G., m., 40 yrs.	Pos.	6½	1 c.c. diluted (1 to 1) blood from Case 2	18	3½	+ +	
(5) P. N., m.?	Neg.	...	9 c.c. diluted (1 to 9) blood, filtered, from Case 2	18	About 29	-	
(6) H. D., m., 45 yrs.	Pos.	15	0.5 c.c. undiluted blood from Case 2	90	6½	+ +	Congenital syphilis
(7) W. J. L., m., 72 yrs.	Neg.	...	2 c.c. diluted (1 to 7) blood, filtered, from Case 4	8	30	-	
(8) J. W. M., m., 63 yrs.	Pos.	10	0.8 c.c. undiluted blood from Case 4	57	99	+ ?	
(9) J. W., m., 65 yrs.	Neg.	...	2.8 c.c. diluted (1 to 3) blood, filtered, from Case 4	57	28	-	
(10) P. M., m., 45 yrs.	Pos.	9	1.3 c.c. washed corpuscles from Case 4	57	28	- ?	

(11)	J. D., m., 74 yrs.	Pos.	7	2 c.c. diluted (1 to 5) citrated plasma from Case 4	57	28	+
(12)	P. B., m., 61 yrs.	Neg.	...	0.3 c.c. undiluted blood from Case 4	130	<24 hours	-
(13)	R. C., m., 58 yrs.	Pos.	8?	2.3 c.c. diluted (1 to 4) citrated plasma, filtered from Case 10	22	49	-
(14)	H. W., m., 70 yrs.	Pos.	5	0.3 c.c. undiluted blood from Case 10	22	49	-
(15)	W. W., m., 30 yrs.	Pos.	7	2 c.c. undiluted serum, filtered, from Case 10	67	22	+ ±
(16)	A. A., m.	Neg.	...	1 c.c. undiluted blood from Case 10	About 190	A few hours	-
(17)	N. K., f., 28 yrs.	Pos.	?	† 2 c.c. diluted (1 to 5) blood, filtered, from Case 11	47	About 48	+ +
(18)	J. F. H., m., 70 yrs.	Pos.	4½	2.6 c.c. diluted (1 to 2½) citrated plasma from Case 11	47	48	-
(19)	T. B., m., 55 yrs.	Neg.	...	2.3 c.c. diluted (1 to 1) serum, filtered, from Case 11	115	70	-
(20)	C. L., m., 70 yrs.	?	?	1.2 c.c. undiluted blood from Case 15	24	About 72	-
(21)	H. McD., m., 42 yrs.	Neg.	...	2 c.c. undiluted blood from Case 20	224	48	-
(22)	P. W. P., m., 71 yrs	Neg.	...	2 c.c. undiluted blood from guinea-pig 3647	See animal experiments		-

\* Hours after onset.—This refers to period elapsing between onset of illness in previous (donor) case and time at which blood was taken from this patient.

† Outside body.—This refers to period elapsing between the time the blood was drawn from donor case and the time when it was injected into the recipient case.



In Case 5 there was some doubt as to whether the result was really negative but there was not a characteristic enough reaction to say that the case was positive. The blood at the time it was taken for filtration was shown to contain the virus (see Case 4). The virus was only outside the body 29 hours.

In Case 7 there was no control to show that the virus was present at the time in the untreated blood from Case 4, but three cases injected with other material taken two days later from the same case (whose blood was used for the filtrate) were positive. It is probable that at the earlier period (8 hours after onset) the blood of Case 4 did contain the virus. The failure in Case 7 cannot therefore be reasonably explained by supposing an absence of the virus at that time in Case 4. The blood was only outside the body 30 hours, and this therefore again cannot be held to account for the failure. The subject will be further considered later.

In Case 9, three cases serve as controls to show that the virus was present in the blood when it was withdrawn from Case 4, and in two of these the virus was outside the body as long as it was in the material used for Case 9 (28 hours) while in the third case the virus was outside the body for a much longer period (99 hours) and was still successfully conveyed.

In the three positive filtration cases—13, 15 and 17—material was taken 22, 67 and 47 hours respectively after the onset of the "mother" cases and was outside the body 49, 22, and about 48 hours respectively.

Before considering the causes determining the failure or success of individual cases we may dwell on the technique of filtration.

### *Technique of filtration.*

The filter used was a small candle about six inches long with a wall one-eighth to one-sixteenth of an inch thick at the top, the inside diameter at the top being ca. one-third of an inch. The filter tapered slightly from top to bottom. It was marked "Chamberland Sme. Pasteur B.E.S.G.D.C.H.B. & Cie, Choisy-le-Roi BES S.G.D.B.," and stamped "Contrôle," and on the bottom "F." It is what is known as the Pasteur-Chamberland F. Filter, which is said to be a coarser grade than the B. type.

For filtration, previously unused candles were fixed in bored rubber corks into the neck of flasks which had side tubes. The whole apparatus was sterilised by steam and cooled, and then attached by the side tube, which was plugged with cotton wool, to the rubber pipe leading to a water pump. The cork was covered with melted paraffin to be sure no leak occurred. The material to be filtered was run into the candle and the pump turned on. When sufficient material had been obtained the plug was removed from the side tube, the end of this flamed and cooled, or wiped with alcohol, and the flask tilted and the filtered material run out into a sterile tube. Generally speaking the pressure at the Health Department Laboratory is not good and the later filtrations were done at the Royal North Shore Hospital Laboratory.

To show that the filters used did not admit the passage of ordinary bacteria, the practice was adopted of mixing organisms with the material to be filtered and testing for their presence by culture in the filtrate.

The organisms added and the nature of the cultural tests were as follow:

Case 5, injected with filtered blood from Case 2 with a doubtful result.

*Organism added.* Emulsion of *B. coli communis* L.I.P.M.

*Cultures.* 1. Broth culture, 1 c.c. of filtrate to 20 c.c. broth. Agar subcultures made from this.

2. Broth cultures, one loop and two loops.

3. Agar cultures, one loop and two loops.

Case 7, injected with filtered blood from Case 4 with a negative result.

*Organism used.* Colon bacillus (*B. acidilactici* type).

*Cultures.* 1. Broth cultures, one and two loops and subcultures from these.

2. Agar cultures, one and two loops.

Case 9, injected with filtered blood from Case 4, with a negative result.

*Organism used.* *B. coli communis* L.I.P.M.

*Cultures.* Broth and agar.

Case 13, injected with filtered plasma from Case 10, with a positive result.

*Organism used.* *B. coli* (type not noted).

*Cultures.* 1. Two loops into 10 c.c. broth.

2. One loop on agar.

Case 15, injected with filtered serum from Case 10, with a positive result.

*Organism used.* *Staphylococcus aureus*.

*Cultures.* 1. Two loops into 10 c.c. broth.

2. One loop on agar.

Case 17, injected with filtered blood from Case 11, with a positive result.

*Organism used.* *Colon bacilli* (type not noted).

*Cultures.* Two loops into 10 c.c. broth.

Case 19, injected with filtered serum from Case 11, with a negative result.

*Organism used.* *Staphylococcus aureus*.

*Cultures.* 1. Two loops into broth.

2. One loop on agar.

All the cultures in this series remained sterile. The number of organisms added to the material before filtration was large but not specially measured. When an emulsion was used it was made densely milky. In other cases a large loopful of thick scrapings from the cultures was used. For full details of this work see under "Experimental Material," following the record of the case from which the material was derived.

Although in future experiments we would be inclined to use larger amounts of the filtrate than two loops for making the cultures, yet we think in view of the number of organisms added that the above tests show fairly clearly that our filters were restraining the passage of ordinary bacteria under the conditions of the experiments. We think the method of adding the organism to the material to be filtered is a much better control than testing the filter before or afterwards, as, with a variable water pressure, it may not be possible to parallel the conditions of the actual filtration. Moreover the filter itself may be altered in some way by washing and sterilising if controlled before the main filtration, and may be blocked by debris from the filtration if the bacterial test is left until afterwards. No serious effects followed the injection

of any of the filtrates, though a slight rise of temperature and some redness of the arm (possibly due to toxins from the bacteria) were noted next day in some cases.

#### FINAL CONSIDERATION OF THE RESULTS OF THE FILTRATION EXPERIMENTS.

Our results indicate that the dengue virus under certain conditions passes through the Pasteur-Chamberland F. Filter, which at the same time is preventing the passage of ordinary bacteria. The question now arises as to what is the cause of the failure in certain experiments.

Confining ourselves to the present series of tests, Case 19 may be rejected as a test as to the filtrability of the virus for reasons given above. In the negative cases, 5, 7 and 9, however, the virus was almost certainly present in the blood on withdrawal and in Cases 5 and 9 was actually shown to be present in the unfiltered blood from which the filtrate was made.

The sojourn outside the body less than 30 hours in any of the cases, is less than in two of our positive filtration cases and in several of our non-filtration positive cases, so that it is at first sight hard to find any ground for the failure of these cases. It is most unlikely that they were all immunes.

The three unsuccessful cases were obtained with filtered blood which before filtration contained a certain amount of solid material and the filtration was done in the Department's laboratory, and although we have no precise notes on the matter, the filtration was certainly slower than in the later cases, including three successful filtrations. Of the later cases, although in Case 13 filtration was also performed at the Department's laboratory, the filtration took place rapidly, the fluid being free from cellular material. In Cases 15, 17 and 19 filtration took place under better pressure conditions at the North Shore Hospital and filtration was more rapid.

We therefore conclude that the failure of certain of our cases was due to slow filtration and the plugging of the filter pores by solid material through which the fluid had to pass, and the successful cases were due to the more rapid filtration brought about by a higher water pressure and an absence of solid material.

#### *Distribution of the virus in the blood.*

Graham (3), whose results have not received confirmation, described endo-corporuscular bodies in dengue fever. These we regard as artefacts. The fact that the virus has been shown to be filtrable, although it shows that at some stage the virus is very small, does not of course exclude the possibility of an endo-corporuscular phase of a microscopically visible size. Although we have examined blood from dengue cases carefully a number of times with unstained preparations, and with the ultramicroscope, we have not found any evidence of a visible virus. Apart from the question, however, of a visible virus it would be useful if possible to show whether the causative agent was confined to any one element of the blood. It is well to remember, however, that the

distribution may not be the same at all stages. The organism may at one stage, for instance, be endo-corpuscular or endo-leucocytic, and at another stage free in the serum. On the other hand the virus may be a special inhabitant of one or other element of the blood.

Our previous results led us to think that, whatever be the nature of the virus, it was apparently not strictly endo-corpuscular, though we could not exclude the possibility of an endo-corpuscular or endo-leucocytic phase followed or accompanied by a phase in which it was free in the plasma. Our present series of experiments, though not yet completely demonstrative, tend to support our previous view.

It should be remembered, however, that processes designed to effect a separation of the blood elements may at the same time cause some breaking up of the corpuscles and a consequent liberation of a virus. A consideration of the whole circumstances, however, leads us to think that this theoretical breaking up cannot explain the results and that, whether or not the virus is solely endo-corpuscular or endo-leucocytic at some stage, it was present in the plasma in the cases examined.

Cases 10, 11, 13, 15 and 18 of the present series may be considered in this connection. For full details of the preparation of the materials injected we refer to the antecedent cases.

Cases 10 and 11 may be first considered together. Case 10 was injected with 1.3 c.c. of four-times washed corpuscles (see Case 4), and Case 11 with the corpuscle-free washings from the same case from blood taken at the same time. Both Cases 10 and 11 were definitely positive.

The 2 c.c. of washings in Case 4 would roughly correspond to the plasma obtained from 0.3 c.c. of the original blood. This dose is small. The amount of corpuscles injected into Case 10 on the other hand was rather large. These two cases seem to us rather to support the contention that the virus is free in the plasma. The large dose of corpuscles used, and the probably great difficulty of freeing such material from adherent virus, may be the explanation of the success with the corpuscles. We unfortunately had no further opportunity of checking these results by injecting more nearly equivalent doses of the two materials.

The other experiments all concern the presence of virus in the fluid element (serum or plasma), and tend to support the view that the virus is naturally free in the fluid element.

Case 18 was injected with "washings" from Case 11, with a positive result. The dose of washings (which showed some haemolysis) was 2.6 c.c., which would be equivalent to the plasma from 0.5 c.c. of the original blood.

Cases 13 and 15, although not specially designed for the purpose, support the finding of virus in the fluid part of the blood. In Case 13 we obtained a positive result after injection of 2.3 c.c. of filtered citrated plasma from Case 10. The dose corresponds approximately to the plasma from 0.23 c.c. of the original blood. Haemolysis was not noted. In Case 15, 2 c.c. of filtrate

from the serum of Case 10 produced a positive result. This serum filtrate was distinctly haemolysed and the dose (2 c.c., undiluted) was large.

#### THE INCUBATION PERIOD.

Our previous results showed that the incubation period of dengue varied between 5 and  $9\frac{1}{2}$  days. We found no incubation periods as short as those described by Ashburn and Craig(4), and by Graham(3). The present series confirms the view that the incubation period is usually about a week, but we have a greater variation in the periods than we encountered previously. However, our shortest period is still considerably longer than the incubation period found by these other observers in Syria and the Philippines.

The incubation periods observed in the successful cases were as follows: 8,  $6\frac{1}{2}$ , 15, 10, 9, 7, 8, 5, 7,  $4\frac{1}{2}$  days (in Case 17 the exact period could not be determined).

The shortest incubation period was actually about 4 days 19 hours (Case 18), and the longest almost exactly 15 days.

There does not seem to be any relation between the time the virus was outside the body and the incubation period, nor between the duration of illness of the previous case and the incubation period.

The cause of the variation is not known.

#### *Immunity.*

There is only one experiment bearing on immunity, namely, Case 3. In this instance the subject of the experiment was injected at the same time, with an approximately equal amount of the same material, as was used to inoculate Case 2 (a non-immune). Whereas Case 2 developed typical symptoms, Case 3 had at no time subsequent any signs of the disease.

Case 3 was the subject of mosquito experiments referred to in our earlier communication(1) and had passed through a typical attack of rather severe dengue starting on 31. v. 16. On 15. i. 17, at 4.15 p.m., Case 3 was inoculated subcutaneously with about 0.5 c.c. of diluted blood from Case 1, and thereafter showed no signs of the disease. Thus 7 months and 15 days (229 days) after the onset of an attack of dengue immunity was present to a dose of virus, which brought about a typical and rather severe result in the control case.

#### PART II. MOSQUITO EXPERIMENTS.

Although a number of experiments were attempted with various species of mosquitoes, *Stegomyia fasciata*, *Culex fatigans*, *Culicella vigilax*, *Culicella annulirostris* and *Scutomyia notascripta*, most of these were failures because the mosquitoes died out or would not bite, or because volunteers were not available. Special experiments with *S. fasciata*, hatched from larvae brought from Mullumbimby, similarly failed. The following experiments, however, are quoted in detail.

*With Culex fatigans.* 22 ♀♀ and 1 ♂ of this species were collected in Sydney and enclosed in a cage with a muslin sleeve. They were allowed to feed on Cases 10 and 11 on 15 and 16. II. 1917, and bit both patients well. Case 10 was thus bitten three or four days after the onset, and Case 11 five and six days after the onset. Case 10 was shown to have virus in the blood a few hours before the biting on 15. II. (see Case 15). Case 11 was bitten rather late and probably the blood at this time did not contain any virus. Many of the mosquitoes were alive on 18. II., but could not be counted owing to the nature of the box. On 18. II. and each night until 26. II. (inclusive) the mosquitoes remaining alive bit Volunteer Mrs W. G. A. No result followed. Although it is not possible to say exactly how many mosquitoes bit either the donor or recipient, it is certain that both were bitten by the mosquitoes in the cage.

The experiment tends to support previous experiments which failed to demonstrate that *Culex fatigans* is capable of transmitting dengue.

*With Culicella vigilax.* About 40 of this species were caught at Berowra on 15. II. 1917, and on the same day bit Case 10 (three days after the onset). Again on 16. II. (four days after the onset) at midday he was bitten. The mosquitoes also bit Case 11 on the same day (five days after the onset) and on 16. II. (six days after the onset).

There were only about a dozen mosquitoes left alive when on 17. II. they were fed on a volunteer, H. McD.—one of the insects bit. After this but few were alive and none bit although tested daily until 21. II. for periods of 30–45 minutes.

No results followed. The experiment is inconclusive.

*Summary.* Several mosquito experiments in this series have not therefore added to our previous knowledge, but the one satisfactory experiment with *Culex fatigans* tends to support the view we hold that this species is probably not a vector of the disease. The results previously published by us support the opinion of Bancroft (5) and show clearly that, whether or not *C. fatigans* may also play a part in Australia, *Stegomyia fasciata* is capable of spreading the infection of dengue.

### PART III. ANIMAL EXPERIMENTS.

#### *An Attempt to Transmit the Virus through a Guinea-pig to a Human Being with a Negative Result.*

On 13. II. (at 3 p.m.) blood was taken from Case 10 (22 hours after the onset). This blood was shown, by the successful injections of Cases 13 and 14, to contain the virus.

On 13. II. (afternoon) a guinea-pig, No. 3647, was injected subcutaneously with 0.25 c.c. of blood (serum and corpuscles). The animal remained well and on 21. II. (morning) it was bled to death, and at noon on the same day 2 c.c. of the blood (serum and corpuscles) were injected into Case 22, P. W. P.,

m., 71 years. His temperature and pulse were taken daily for four days, and four-hourly for 14 days.

On 25. II. at 10 a.m. (four days after the injection) the temperature reached 99° F., and was on the same level during part of the day of 26. II., but this was unaccompanied by any other signs or symptoms, and the temperature after this remained normal.

There is no evidence, therefore, of the survival of the dengue virus after 7½ days in a guinea-pig or of its multiplication in this animal.

#### OTHER ANIMAL EXPERIMENTS.

These need not be separately detailed as they were uniformly negative.

Guinea-pigs and rabbits were injected intraperitoneally and subcutaneously without result, and sections from the organs of some of these were examined both by iron-haematoxylin and eosin stained sections and by Levaditi's method, and showed no abnormality. Our object in using Levaditi's method was in the hope of demonstrating possible spirochaete-like organisms and was undertaken in view of the results obtained with epidemic jaundice in France.

#### APPENDIX

##### DETAILED REPORT OF THE CASES DEALT WITH IN THE SERIES OF INJECTION EXPERIMENTS.

**Case 1, Dr F. G. (naturally infected).** This case forms the starting-point of the series of experiments with which this report is concerned.

The patient was on a holiday in Molangool, near Bundaberg, Queensland, where he states that occasional cases of dengue fever were occurring, and where mosquitoes (*Stegomyia fasciata*) were biting freely in the daytime. He left Molangool on 5. 1. 17, passing the night in Bundaberg and thence journeying to Sydney by train. He arrived in Sydney on the evening of 7. 1. 17. Thus his last day in Queensland, and the last day on which he was exposed to *Stegomyia* bites, was on 6. 1. 17. He became ill on 11. 1. in the evening, therefore the incubation period must have been at least five days, and, assuming he was infected in the Molangool district, six days or more.

He kindly volunteered to allow blood to be taken for our experiments, and accordingly one of us went to see him on the 13. 1. at 4 p.m., and took about 1 c.c. of blood from the median basilic vein.

This case was apparently typical and fairly severe. He had the usual symptoms and a marked rash, which was very obvious on 13. 1. He had not taken his temperature regularly and only stopped in bed one day.

**Experimental material.** The blood taken on 13. 1. 17, at 4 p.m., ca. 46 hours after the commencement of the illness, was kept on ice until 15. 1. at 4 p.m., when it was used to inject Cases 2 and 3. There was about 1 c.c. of clot and serum in the test-tube. This was shaken up with about 2 c.c. of sterile normal saline solution, and about 1 c.c. of the fluid part, containing

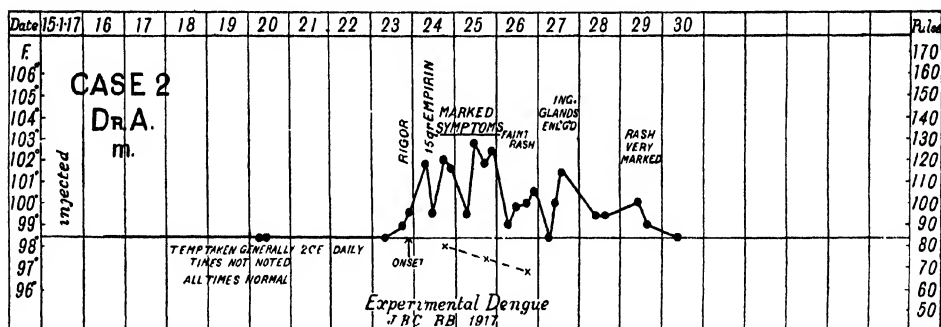
corpuscles, serum and saline, was sucked up into a syringe and equal amounts were injected into Cases 2 and 3.

Subcutaneous injection of 0.5 c.c. of diluted (1 to 2) blood taken from Case 1, 46 hours after the onset, virus kept outside the body 48 hours. *Result, positive.*

**Case 2, Dr W. G. A., m.** 15. I. 17. On this date Dr A. of this Department volunteered for experiment, and at 4 p.m. he was injected subcutaneously with 0.5 c.c. of diluted blood from Case 1. The blood had been taken from Case 1 about 46 hours after the onset and had been outside the body for 48 hours; it was diluted ca. 1 to 2.

From this date the temperature, which was taken once at least and usually twice daily, remained normal until 23. I.

20. I. On this date he first felt slight rheumatic pains and some degree of languor on rising in the morning. The temperature was normal at 7 a.m.,



and at 9.30 a.m. There is no record of the evening temperature. After this there were occasionally some vague symptoms of languor, etc., but no rise of temperature until 23. I. Leucocytes 8500 per c.mm.

23. I. At 5 p.m. the temperature had risen to 99° F., and by 9 p.m. to 99.5° F. At 10 p.m. there was a rigor. A restless night was passed and he had occipital headache, painful eyes and vague rheumatic pains.

24. I. At 7.30 a.m. the temperature was 101.8° F. The patient came in to work and between rising and 11.30 a.m. took three 5-grain doses of aceto-salicylic acid. The temperature was 99.5° F. at 11.30 a.m. At midday he returned to his home. At 5.30 p.m. the temperature was 102° F. and the pulse 80, and at 9.30 p.m. the temperature was 101.6° F. At about 11 a.m. blood had been withdrawn from a vein and used to inject Cases 4 and 5. Blood from the ear and the serum from clotted blood from the vein were examined ultra-microscopically without finding any recognisable parasites.

25. I. He stopped in bed all day and had severe headache, pains in the limbs and eyes, and several chills. He took 5 grains of aceto-salicylic acid every three hours. The temperature at 7.30 a.m. was 99.5° F.: at 11.30 a.m.



102·8°; at 5.30 p.m., 101·8° (pulse 74); at 9.30 p.m., 102·4°. He passed a very restless night and did not sleep until 4.30 a.m.

26. i. A faint macular rash was discernible on the abdomen and thorax. The temperature ranged from 99° to 100·5° F. (see Chart).

27. i. The inguinal glands were distinctly enlarged on both sides. The temperature was 100° F. at 10.30 a.m., and 101·4° at 2.30 p.m. Leucocytes 3400 per c.mm. Blood was taken on this day about 11 a.m. from a vein and used to inoculate Case 6.

28. i. There was a faint but distinct morbilliform rash on the hands and forearms, most distinct on the palms. The temperature was 99·4° F. morning and evening.

29. i. The rash was very distinct and well-defined on the abdomen, chest, forearms, thighs, back, and palms of the hands. The face was free. The temperature was 99° to 100° F.

30. i. The temperature was normal and the patient felt practically well, although stiffness and a slight feeling of indisposition were present for some days afterwards.

*Remarks.* The case was a very definite one of dengue of rather severe type and the patient looked very ill for several days. The rash was extremely marked and very widespread. The incubation period, to the rise of temperature, was almost exactly eight days, but vague indisposition occurred just before five days had elapsed. Blood from this case, taken on the second day after the rise of temperature (24. i. 17), ca. 18 hours after the onset, gave a *positive result*, i.e. it induced the typical disease in Case 4, and blood taken on the fifth day of the disease (27. i. 17), or about 90 hours after the rise of temperature, caused the disease in Case 6.

*Experimental material.* (a) Blood was taken at 11 a.m. on 24. i. 17, ca. 18 hours after the onset as indicated by a rise of temperature. About 2·5 c.c. of blood was thus obtained and allowed to clot, and then ground up in a mortar with 2·5 c.c. of sterile normal saline solution.

1 c.c. of the more fluid part of this mixture was injected into Case 4.

1 c.c. of the same was injected into a guinea-pig, without result.

3 c.c. of the same was mixed with 2 c.c. of a saline emulsion of *B. coli communis* and filtered through a Pasteur-Chamberland F. candle and subsequently 10 c.c. of water were added. About 10 c.c. of red stained fluid came through fairly quickly. There remained in the filter candle about 5 c.c.

The filtrate was tested as follows:

On 24. i. 17, 1 c.c. was added to about 20 c.c. of broth. The original culture remained sterile up to 31. i., after which it was not further examined. Agar subcultures from this remained sterile. The last subculture was made on 31. i. and examined on 1. ii.

On 24. i. 17 broth and agar cultures were made with one or two loops of the filtrate. These remained sterile until the last examination on 31. i.

The remaining filtrate (ca. 9 c.c.) was used to inject Case 5.

Although the above figures for dilution are only approximately accurate, it will be observed that the blood used to inject Case 4 was diluted ca. nine times with saline and water (1 to 9).

(b) Blood taken at 11 a.m. (90 hours after the onset) on 27. i. was not treated in any way but was used to inject Case 6.

Injection of a volunteer who had had dengue eight months previously, with virulent dengue blood. *Result negative.*

**Case 3, Dr B. B., m., 34 years.** One of us, who had been the subject of experimental mosquito-borne dengue eight months previously, was injected on 15. i. 17, at 4 p.m., with 0.5 c.c. of the diluted blood from Case 1. The amount injected was approximately equal to that injected at the same time into Case 2.

On 16 and 17. i. there was some headache, sore throat and languor but no areola round the injection and the temperature was normal. No symptoms developed after this date.

The result was *negative*, but Case 2, similarly injected, but not protected by a previous attack of dengue, developed this disease.

Subcutaneous injection of 1 c.c. of about equally diluted blood and saline solution taken from Case 2, 18 hours after the onset, virus kept outside the body  $3\frac{1}{2}$  hours. *Result positive.*

**Case 4, H. G., m., 40 years.** This volunteer was injected subcutaneously on 24. i. 17, at 2.30 p.m. with 1 c.c. of diluted blood serum and corpuscles from Case 2. The blood was taken from Case 2 at 11 a.m. on 24. i. 17 (18 hours after the onset). The dilution was with approximately equal parts of blood and sterile saline solution (for details see Case 2). The virus was outside the body about  $3\frac{1}{2}$  hours.

The patient remained well until 31. i. although he complained of slight headache a day or so before the definite onset.

31. i. 17. He arrived at the Laboratory looking very sick and said he had been taken ill about 3 a.m. He had pains "all over" and headache and felt very weak. His face was flushed. There was no definite rash. The temperature was  $100.4^{\circ}$  F. and the pulse 120, on arrival at the Laboratory about 10 a.m. The leucocytes were 8700 per c.mm.

Blood was taken on this day at about 11 a.m. from the median basilic vein and, after filtration, was used for the injection of Case 7, with a *negative result*.

2. ii. He stated that he had had two "bad" days at home, feeling sick, but was better now. There was a well-marked rash. Leucocytes 16,000 per c.mm.

Blood taken at midday from a vein was used as material to inject Cases 8—11, three of which were *positive*.

5. II. The patient felt fairly well.

Blood was taken at midday (?) and used to inject Case 12 with a *negative result*.

*Remarks.* The incubation period was  $6\frac{1}{2}$  days. Although a temperature chart was not regularly taken, the case was absolutely typical and moderately severe. The rash was well marked on the trunk and arms. Inoculation with blood taken from this case, 31 hours after the onset, gave a *positive result*, reproducing the disease in several cases.

*Experimental material. First specimen.* A specimen of blood was drawn from a vein on 31. I. 17, at about 11 a.m., about eight hours after the onset. A little serum was separated for the Bordet-Gengou test for syphilis, which proved to be negative.

The remaining serum and clot were mixed with about equal parts of boiled tap water. Some of this mixture was removed with the object of using it subsequently for injection but when required it was found to have become contaminated. The remainder was again equally diluted with a watery emulsion of a colon bacillus (*B. acidi lactici* type) and crushed up as far as possible in a mortar. The more fluid portion was put into the filter but would not pass through the candle. A further addition of water of approximately equal amount to the already diluted material was added. Filtration was slow and only about 2 c.c. of filtrate were obtained. The dilution, when filtered, was about 1 to 7, but this is not accurate having regard to the removal of the more fluid part for the Bordet-Gengou reaction, etc., and the final discarding of the clot.

Cultures were made from the filtrate—one and two loops on broth, and one and two loops on agar, and subsequently subcultures were made on agar from the broth. All cultures remained sterile.

The whole filtrate, consisting of about 2 c.c. of fluid, was injected into Case 7 with a *negative result*.

*Second specimen.* This was taken from a vein on 2. II. 17, at midday, ca. 57 hours after the onset.

(A) 2 c.c. were squirted from the syringe into ca. 10 c.c. of 1·5 % sodium citrate in normal saline. This was centrifuged for 15 minutes and the supernatant fluid separated from the deposit.

The *supernatant fluid* was then centrifuged for one hour and the fluid, down to  $\frac{1}{2}$  in. from the bottom of the tube, drawn off. This was labelled "*washings*," and contained no corpuscles microscopically. The dilution would be 1 to 5. Cultures on broth and agar made on 2. II. remained sterile. These "*washings*" gave a *positive result* in Case 11.

The *corpuscles* from the first centrifugalisation were suspended in sterile saline solution and shaken and recentrifuged for 15 minutes. The supernatant fluid from this centrifugalisation was drawn off, fresh sterile saline added, and the mixture again shaken and recentrifuged for 15 minutes. The procedure was repeated and the residual corpuscles were retained. Cultures made on

agar and broth remained sterile. These four-times washed corpuscles were labelled "*washed corpuscles*." They gave a *positive result* in Case 10.

(B) About 5 c.c. of blood-clot were broken up with a sterile wire, and 2 c.c. of the more fluid part were separated and labelled "blood"; 0.8 c.c. (14 minims) of this were used to inject Case 8, with a *positive result*.

(C) The remaining 3 c.c. of clotted blood were diluted with about 9 c.c. of a watery emulsion of *B. coli* L.I.P.M., and were filtered in the usual way. The filtrate was tested by agar and broth cultures and found sterile. This was labelled "*filtrate*," and 2.8 c.c. were used to inject Case 9 with a *negative result*.

*Third specimen.* Blood was taken on 5. II. 17 at ? midday, and without dilution, 0.3 c.c. (5 minims) of serum and corpuscles were injected into Case 12. With *negative result*.

*Summary of experimental results obtained from material from Case 4.*

Diluted (1 to 7) filtered blood taken about eight hours after the onset gave a *negative result* (Case 7).

Undiluted blood taken 57 hours after the onset gave a *positive result* (Case 8).

Washed corpuscles taken 57 hours after the onset gave a *positive result* (Case 10).

Diluted (1 to 5) washings taken 57 hours after the onset gave a *positive result* (Case 11).

Diluted (1 to 3) filtered blood taken 57 hours after the onset gave a *negative result* (Case 9).

Undiluted blood taken approximately 130 hours after the onset gave a *negative result* (Case 12).

Subcutaneous injection of 9 c.c. of 10 times diluted filtrate (1 to 9) from Case 2, taken less than 18 hours after the onset. Virus kept outside the body ca. 29 hours or less. *Result negative*.

**Case 5, P. N., m.** On 25. I. 17, this volunteer was injected, at a time not noted but probably about 4.30 p.m., with 9 c.c. of a filtrate prepared from blood taken from Case 2 on 24. I. 17, at 11 a.m., *i.e.* ca. 18 hours after the onset. The virus was thus outside the body probably about 29 hours. This dilute filtrate would correspond roughly to the fluid from 0.9 c.c. of blood. The arm was sore and showed an erythematous flush for a few days around the site of inoculation, and the temperature rose next day to 99° F. This rise was evidently due to toxins in the material inoculated, probably from the colon bacilli used as a test for possible permeability of the filter.

Examination of the temperature chart (not published), which was kept from 28. I. until 28. II. 17, and was taken four-hourly from 3 to 28. II., shows a rise to 99.4° F. on the ninth day, followed by several minor rises

above normal, until on the 22nd day the temperature reached 100° F. followed by 99·2° F. on the 23rd and 24th days of illness.

During the whole period that this chart was kept, the patient worked hard and felt well. His body was examined daily but showed no rash or other signs of infection. The pulse was variable, being usually rather slow and often at about 50 and sometimes lower.

*Remarks.* Taking all things into consideration, although it is possible that there may have been a modified reaction to the virus, the case must, for the purposes of proof of filtrability of the dengue virus, be regarded as *negative*. This experiment is discussed separately in the section dealing with filtrability of the virus.

Subcutaneous injection of 0·5 c.c. of undiluted blood from Case 2, taken 90 hours after onset. Virus kept outside the body 6½ hours.

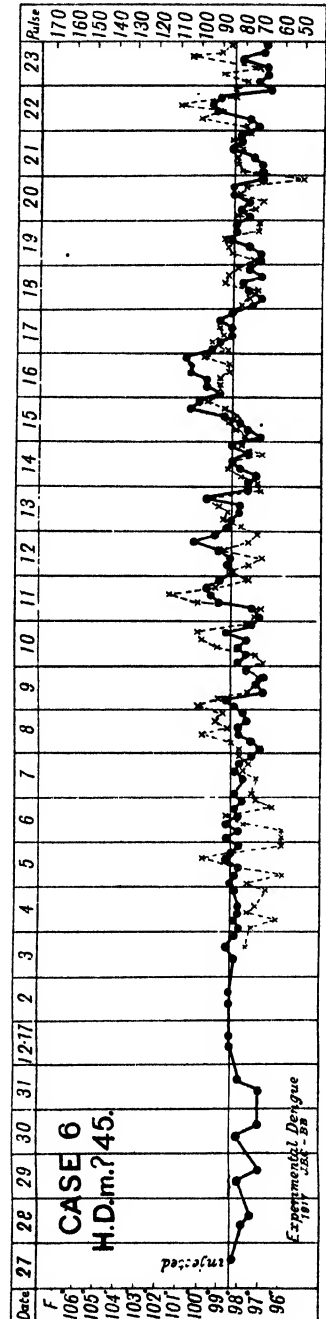
*Result positive.*

**Case 6, H. D., m., 45 years.** This case was injected at 5.30 p.m. on 27. I. 17 with 0·5 c.c. of untreated blood taken from Case 2 on 27. I. 17, at 11 a.m. The virus had been taken 90 hours after the onset and was outside the body 6½ hours.

This patient felt a little "out of sorts" on the afternoon of 11. II. 17, and had a rash on his shoulders with a temperature at 6 p.m. of 99·6° F.

12. II. 17. On examination the face was suffused, the eyes and pharynx injected, the tongue slightly coated, and a slight cough was present. There was a well-marked rash consisting of a pinkish raised papular erythematous mottling over the whole body, except below the knees and on the hands. The rash tended to run together into large patches. It was very copious in the armpits and groins. It began on the shoulders and quickly covered the upper three-fourths of the body.

13. II. The rash was more copious but



slightly duller in colour. The distribution was the same. The rash was seen by several medical men and photographed. The patient felt well.

14. II. The rash was fading. There was still some cough. The patient felt well.

15. II. The same as on 14. II.

16-17. II. The rash was only visible on the back. The patient felt well.

18-21. II. There was now only an erythematous blush on the back and no rash. The patient felt well.

23. II. The patient was allowed up.

*Remarks.* The chart of this case shows an irregular diphasic temperature variation with a late third rise. The incubation period, measured to the first definite rise of temperature, is about 15 days, which is double that of the usual period found in our first series of cases. However, the present series contains other instances of long periods and the case cannot be rejected as being positive on this account. The symptoms were very mild but the patient was of a very low mental type and it was hard to get intelligent replies to questions.

A specimen of blood was taken with the object of using it for further inoculations, but the case gave a strongly positive Bordet-Gengou ("compluetic") test and thus prevented this being done. The rash was in our opinion, and in that of others who saw it, definitely not a syphilitic manifestation and equally definitely was of the type seen in dengue. The case bore evidence of old, probably congenital, syphilis and no evidence of recent infection.

The temperature-pulse relationship, although not typical of dengue as seen in some of the charts, shows for the most part a relatively slow pulse in relation to the height of temperature.

Subcutaneous injection of 2 c.c. of filtered blood from Case 4, the blood taken eight hours after the onset in Case 4. Virus kept outside the body 30 hours. *Result negative.*

**Case 7, W. J. I., m., 72 years.** On I. II. 17, at 4.30 p.m., the volunteer received subcutaneously 2 c.c. of the filtrate from Case 4. The blood from which this was obtained was taken on 31. I. 17, eight hours after the onset, and was kept outside the body for 30 hours. The temperature was charted for 22 days twice daily and, except for a rise to 99° F. on the day after the injection, remained normal.

*Remarks.* This case gave a definitely *negative result* with filtered diluted blood. There was no control case injected with untreated blood taken on this date, but blood taken on the third day of Case 4 gave *positive results*, and therefore it is almost certain that blood taken on the first day was infective and that the failure of the filtrate to produce infection cannot be attributed to an absence of virus in the blood.

This case is discussed fully in the section on filtrability of the virus.

Subcutaneous injection of 0.8 c.c. of untreated blood taken from Case 4, 57 hours after the onset. Virus kept outside the body 99 hours. *Result positive.*

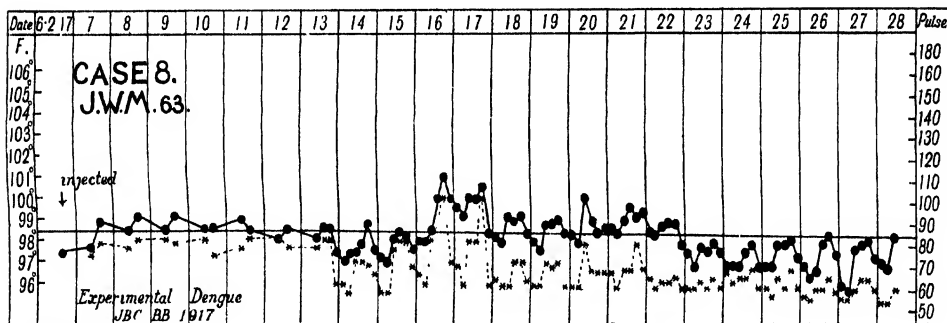
**Case 8, J. W. M., m., 63 years.** On 6. II. 17, at 3 p.m., the case received subcutaneously 0.8 c.c. of blood which had been withdrawn from Case 4 on 2. II. 17 at noon, that is, 57 hours after the onset, and kept on ice for 99 hours.

The temperature, as can be seen on the chart, was slightly above normal for several days after the injection, but between 11. II. and 15. II. it remained practically normal or subnormal.

16. II. When the patient entered the ward at 2 p.m. to have his temperature taken, he looked flushed, and his temperature was found to be 100° F., but he did not complain of any symptoms. That evening the temperature rose to 101° F.

17. II. He was examined in bed: face flushed, eyes clear, tongue not coated, pharynx injected, slight cough but no coryza. He stated he felt well. There was an erythematous flushing of the back. Maximum temperature 100.5° F.

18-19. II. No rash present, tongue coated. Temperature was 99.2° F.



20. II. Tongue clearing, a faint mottling on the abdomen and back. Maximum temperature 100.2° F.

21. II. Mottling on chest, back and abdomen, but not on legs and arms. The patient felt well. Temperature 99.7° F.

27. II. Patient allowed up. The rash was fading. Temperature had been subnormal since 23. II.

**Remarks.** The incubation period was ten days. The temperature variation was roughly diphasic having its maxima at 6 p.m. on 16. II. and at 10 a.m. on 20. II. The pulse tended to be slow and this bradycardia is noticed on the chart to start several days before the definite onset. During the first period of pyrexia the pulse was on two occasions raised correspondingly with the temperature, but for the most part and especially in the later stages of the pyrexia remained relatively slow, until the end of the temperature-taking.

Although slow, there was a noticeable correspondence between the oscillations of the pulse and those of the temperature, a correspondence which is seen in several cases. The most striking feature was the entire absence of symptoms. There can be little doubt, however, that this case was one of mild dengue infection.

The patient gave a partially positive Bordet-Gengou (complotic) reaction, and therefore further inoculations could not be practised.

Subcutaneous injection of blood filtrate from Case 4. The blood was taken 57 hours after the onset and was kept outside the body 28 hours. *Result negative.*

**Case 9, J. W., m., 65 years.** On 3. II. 17, at 4.30 p.m., 2.8 c.c. of filtrate (diluted 1 to 3) of blood from Case 4 taken on 2. II. (noon), 57 hours after the onset, were injected subcutaneously. The virus had then been outside the body for 28 hours.

The temperature was taken twice daily for seven days and thereafter four-hourly for 14 days. There was a rise to 99.4° F. on 7. II., and there were slight oscillations later not exceeding 99°, but insufficient to indicate infection. There were no symptoms, the patient feeling perfectly well during the whole period.

*Remarks.* Result negative. There were three other cases (8, 10, 11) inoculated with unfiltered preparations of blood taken at the same time, all of which were *positive*.

The case is fully discussed under the section dealing with filtrability.

Subcutaneous inoculation with 1.3 c.c. of washed corpuscles from Case 4.

The blood was taken 57 hours after the onset, and was kept outside the body 28 hours. *Result positive.*

**Case 10, P. M., m., 45 years.** On 3. II. 17, at 4.30 p.m., 1.3 c.c. of washed corpuscles from Case 4 (*q.v.*) were injected subcutaneously into this case. The blood from which the corpuscles were derived had been taken from Case 4 on 2. II. 17, at noon, that is, 57 hours after the onset. The virus was kept outside the body 28 hours.

12. II. 17. At 5 p.m. he became suddenly ill with severe frontal headache and flushing. During this night he felt hot, flushed and headachy. The temperature rose steeply in the evening, reaching 101° F. at midnight.

13. II. Face flushed, eyes injected, tongue coated, no coryza. He stated that the headache had now practically gone. There was an erythematous flushing of the back but no rash. Temperature varied between 100° and 101° F. Blood was taken for experimental purposes. The Bordet-Gengou (complotic) test was negative.

14. II. Face still flushed, eyes injected, tongue coated. He felt well. A faint mottling on the back. Temperature varied between 100° and 101° F.



15. II. Temperature lower, the highest point reached being 100° F. There was still faint mottling on the back. The patient felt well. Another sample of blood was taken.

16. II. Maximum temperature 100.2° F. Mottling still visible on the back. The patient felt well.

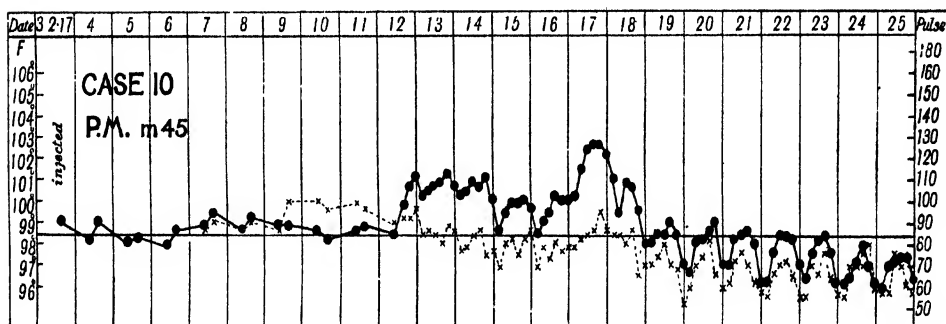
17. II. Rash still present on back and faintly marked on chest. He complained of feeling hot. Temperature rose to 102.8° F.

18-19. II. He did not feel so well. Temperature fell from 102° F. to subnormal.

20. II. He felt well. Maximum temperature 99° F. A third specimen of blood was taken.

After this the temperature did not rise above 99° F., and after 21. II. 17 was normal or subnormal. He was allowed up on 22. II. The rash was still present but he felt well.

*Remarks.* The inoculation undoubtedly caused a definite attack of dengue. The incubation period was nine days. The temperature variation



was definitely diphasic of the saddle back type. The pulse showed definitely the failure to respond proportionately to a rise in temperature so frequent in these cases. There was relative bradycardia from the beginning to the end of the period of pyrexia, and after the attack, the pulse was periodically absolutely slow. In this case we note again a rough correspondence in the chart between the oscillation of the pulse and that of the temperature, although the pulse is throughout the illness slow.

It will be noted that prior to the onset there was a period during which the pulse rate was higher than usual, but as soon as the pyrexia appeared the pulse became slower, only rising during the second part of the pyrexia to 95, although the temperature was 102.8° F.

Blood from this case, taken 22 hours and 67 hours after the onset, reproduced the disease, but blood taken eight days (ca. 190 hours) after the onset gave a negative result.

*Experimental material. First specimen.* Blood was withdrawn on 13. II. 17, at ca. 3 p.m., i.e. ca. 22 hours after the onset.

(a) 2 c.c. of this was squirted into about 8 c.c. of sterile 1·5 % sodium citrate in normal saline solution. This mixture was centrifuged free from corpuscles and filtered in the usual way after adding scrapings of an agar culture of *B. coli*. Filtration was rapid, ca. 5 c.c. of fluid being obtained in a few minutes. The dilution was 1 to 4.

Cultures were made by adding two loopfuls of filtrate to 10 c.c. of broth and also by smearing a loopful on agar. These cultures remained sterile.

2·3 c.c. of the filtrate were used to inject Case 13 on 15. II. 17 at 4 p.m. with *positive result*.

(b) Serum from the clotted blood gave a negative Bordet-Gengou (comphetic) reaction.

Accidentally most of the clotted blood was thrown away, but there were a little serum and corpuscles left in the test-tube and these, after being shown to be sterile by culture, were injected into Case 14 on 15. II. 17 at 4 p.m. About 0·3 c.c. of blood was injected with *positive result*.

*Second specimen.* A large sample of blood, about 20 c.c., was withdrawn on 15. II. 17, at about midday, ca. 67 hours after the onset.

About 10 c.c. of serum were freed from corpuscles by centrifugalisation; scrapings of a culture of *Staphylococcus aureus* were added and the material filtered. Filtration was rapid. Cultures on broth (two loops) and agar (one loop) were sterile. 2 c.c. of the filtrate were used to inject Case 15 with a *positive result*.

*Third specimen.* Blood was taken on 20. II. 17, allowed to clot, and 1 c.c. of serum and corpuscles were used that day to inject Case 16, with *negative result*. This specimen was taken from Case 10 about 190 hours after the onset.

Subcutaneous injection of 2 c.c. of "washings" from Case 4, taken 57 hours after the onset. The material was kept outside the body for 28 hours.

*Result positive.*

**Case 11, J. D., m., 74 years.** On 3. II. 17, at 4.30 p.m., J. D. was injected subcutaneously with 2 c.c. of (1 to 5 diluted) corpuscle-free washings from the citrated blood of Case 4, the blood was taken on 2. II. at noon, 57 hours after the onset, the virus having been kept outside the body for 28 hours.

8. II. He complained of languor and some diarrhoea. Temperature normal.

9. II. He said he felt better but was "tired." Temperature normal.

10. II. Temperature rose to 99·0° F. at 4.25 p.m. During the night he felt so giddy and headachy that he was put to bed. (He was employed as night-watchman.) Temperature rose to 101·6° F. by midnight.

11. II. Temperature at 4 a.m. 102·4° F., ca. 101° to 102° during the day.

12. II. Face flushed, eyes and pharynx injected, tongue coated. There was pain in the neck and lumbar region and slight cough but no coryza. There was no rash. The patient complained of sleeplessness. Blood was taken on

this day and gave a *positive* result in the form of a filtrate in Case 17 and as "washings" in Case 18.

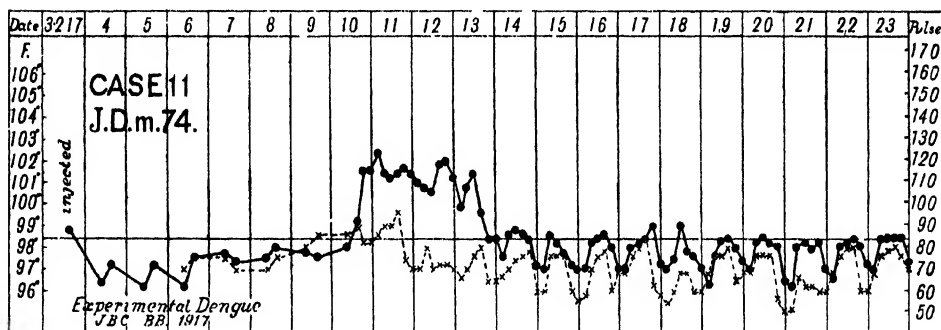
13. II. He felt better and there were no pains. There was some suggestion of a rash. Temperature fell to normal.

14. II. A pinkish erythematous mottling over the whole back, chest, abdomen, thighs, shoulders and arms, but nothing on the legs, feet or hands. He felt well. Temperature about normal.

15. II. The rash fading. Blood taken on this date gave a *negative* result as a serum filtrate injected into Case 19. The temperature was normal at this time.

16-22. II. The rash faded gradually and the patient felt quite well. He was allowed up on 22. II. There were rises of temperature on 17 and 18. II. to 99° F.

26. II. He complained of weakness. The temperature had been normal or subnormal since 18. II.



*Remarks.* The incubation period, measured to the first rise of temperature, was seven days. The previous symptoms are regarded as premonitory. The chart shows a definitely maintained rise of temperature from 10 to 13. II. inclusive. On the evening of 13. II. the temperature fell sharply and thereafter, except for two isolated rises to 99° F. on 17 and 18. II., was normal or subnormal until 23. II. (end of chart).

The pulse-temperature relationship was "typical." Except for a slight increase on the day after the onset of fever, the pulse failed to respond to the rise of temperature. Relative bradycardia is marked. Towards the end of the chart are seen occasional periods of definite absolute bradycardia. Although the rash was not marked it was definite. *The result was positive.*

*Experimental material. First specimen.* On 12. II. at 3 p.m., 47 hours after the onset, about 10 c.c. of blood were taken from a vein. 7 c.c. of this were allowed to clot—about 1 c.c. of the serum was then abstracted for a Bordet-Gengou (compluetic) test (which proved negative), and also another 1 c.c. of serum and corpuscles was abstracted for injection purposes but not used. The remaining serum and clot (about 5 c.c.) were mixed with about

10 c.c. of a watery emulsion of colon bacilli and shaken well together. After allowing the solid material to deposit, the more fluid part of the mixture was again equally diluted with *tap-water* and filtered in the usual manner through a Pasteur-Chamberland F. filter.

About 5 c.c. of filtrate were rapidly obtained (the candle still retained about 6 c.c. of material). The filtrate was tested by making cultures on agar (one loop) and on broth (two loops), and these remained sterile. The specimen was labelled "blood filtrate, Case 11," and about 2 c.c. were used to inject Case 17 with a *positive result*. The dilution was approximately 1 to 5.

About 2 c.c. of the original blood was mixed with about 5 c.c. of 1.5 % sodium citrate normal saline solution. The mixture was centrifuged for half-an-hour and the supernatant fluid removed. Some haemolysis had occurred and the supernatant fluid still contained some red corpuscles. The supernatant fluid was recentrifuged, poured off, and left overnight. On the next day this was again recentrifuged and the supernatant fluid removed and labelled "washings from Case 11." 2.6 c.c. were used to inject Case 18 with a *positive result*.

We have no note as to the microscopical examination of the final fluid but it is safe to say it was then free from corpuscles. The dilution was approximately 1 to  $2\frac{1}{2}$ .

*Second specimen.* About 15 c.c. of blood were taken on 15. II. 17 at about noon, and the serum was centrifuged free from corpuscles. About 3 c.c. of the serum, distinctly stained with haemoglobin, were obtained and this was diluted equally with water. To the dilute material a loopful of an agar culture of *Staphylococcus aureus* was added. It was filtered in the usual way through a Pasteur-Chamberland F. candle. The filtrate came through rapidly and was labelled "*serum filtrate from Case 11.*" Cultures made on agar (one loop) and broth (two loops) proved sterile. This filtrate was used to inject Case 19 with a *negative result*.

Subcutaneous injection of 0.3 c.c. (5 minims) of untreated blood from Case 4, ca. 130 hours after the onset. Virus kept outside the body less than 24 hours. *Result negative*.

**Case 12, P. B., m., 61 years.** On 6. II. 17, at 10 a.m., he received subcutaneously 0.3 c.c. of serum and corpuscles from Case 4, which had been taken on 5. II. about 130 hours after the onset. The virus was kept outside the body for less than 24 hours.

The temperature and pulse were taken twice daily for six days, and then every four hours for 16 days, but no indications of infection followed.

*Remarks.* The injection gave a *negative result*, this indicating that the virus was not present in the blood of Case 4 on the sixth day (ca. 130 hours) from the onset.

Subcutaneous injection of 2.3 c.c. of citrated plasma filtrate from Case 10.

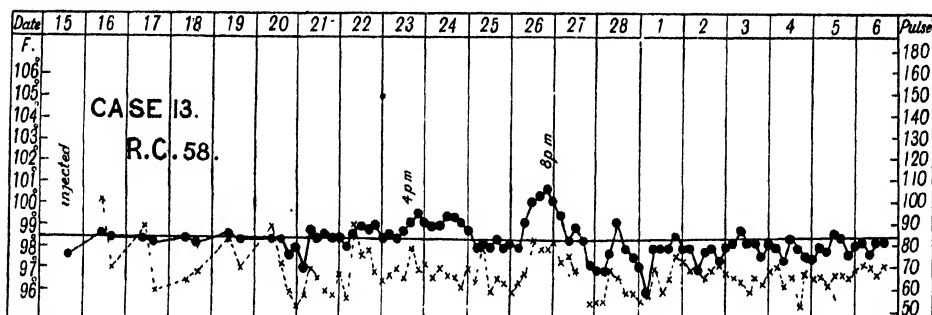
The blood was taken about 22 hours after the onset. Virus kept outside the body 59 hours. *Result positive (mild).*

**Case 13, R. C., m., 58 years.** On 15. II. 17, at 4 p.m., R. C. was injected subcutaneously with 2.3 c.c. of citrated plasma filtrate taken from Case 10. The blood was withdrawn from Case 10 at 3 p.m. on 13. II. or 22 hours after the onset. The virus was kept outside the body for 48 hours.

21-22. II. The temperature was as high as 98.8° F. at 8 a.m. on 21. II. but thereafter remained about normal until midday on 22. II., when it reached 99° F.

23. II. Temperature was 99.2° F. at 4 p.m., and 99.6° at 8 p.m. The patient complained of feeling "out of sorts," and was put to bed for observation.

24. II. Temperature remained at 99° F. during the day, rising to 99.4° in the evening. He had slight headache and depression and also a faint flushing of the face and back.



25. II. Temperature fell and the patient felt well.

26. II. Patient complained of headache during the previous night. Tongue coated, face and back flushed, but there was no rash. During the afternoon and evening, the temperature rose rapidly, reaching 100.8° F. at 8 p.m.

27. II. Temperature subnormal during the morning, and 99° F. at midday.

2. III. Since 28. II. the temperature had been normal. On this date the patient complained of pain in the chest and was not so well.

3. III. Slight headache and flushing of the back were present. Tongue coated.

There was nothing further to note after this date, and the patient got up on 5. III.

**Remarks.** It is hard to fix the time of onset of the pyrexia, as will be seen by reference to the chart. As early as 21. II. there was a rise to nearly 99° F. and during 22. II. the temperature was on the 99° level. On 23. II. the temperature rose definitely, reaching 99.6° F., and on 24. II. the highest point was 99.5° F. On the evening of 26. II. there was a steep rise to 100.8° F.

at 8 p.m., but by 8 a.m. on 27. II. this had subsided to rise to 99° at midday. Thereafter, with the exception of one slight rise to 99·2° on 28. II. the temperature was practically normal.

Considering the occurrence of symptoms, mild but definite, on the evening of 23. II., it is probable that the onset occurred about this time and was followed by the mild attack of dengue. Assuming that the evening of 23. II. represents the beginning of the attack, the incubation period was eight days. The type of chart was definitely diphasic with an initial slight pyrexia, and a secondary more definite pyrexia on 26. II.

The pulse is characteristic in both phases, failing almost completely to respond to the rise of temperature.

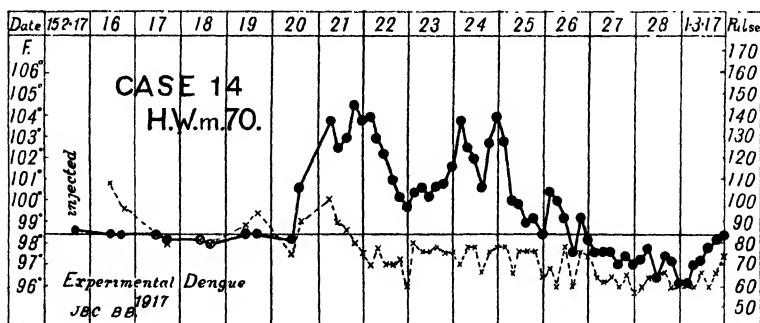
*Experimental material.* It was impossible to utilise the blood from this case for further experiments as there were no volunteers obtainable.

Subcutaneous injection of about 0·3 c.c. of untreated blood (serum and corpuscles) taken from Case 10, about 22 hours after the onset. Virus kept outside the body 49 hours. *Result positive.*

**Case 14, H. W., m., 70 years.** On 15. II. 17, at 4 p.m., H. W. was injected with about 0·3 c.c. of serum and corpuscles taken from Case 10 on 13. II. at 3 p.m., i.e. 22 hours after the onset. The virus was kept outside the body for 49 hours.

20. II. Temperature 100·6° F. at 3 p.m. Patient said he felt "flushed" but he had no other symptoms.

21. II. Temperature 103·8° F. in the morning, and 104·6° in the evening, but the patient said he felt well. Face flushed, eyes injected, tongue coated. A distinct erythematous flushing of the back but no definite rash.



22-23. II. A faint mottling on the back but nothing else to note. Patient felt well. A distinct fall of temperature occurred to 100° F. but it gradually rose again.

24. II. Patient said he did not feel so well. Temperature rose sharply to 103·8° F. in the early hours of the morning, and fell during the day to 100·6° F., rising again at night to 104°.

25. II. Temperature fell rapidly to 99° F. The patient felt well.

26. II. Temperature rose to 100.4° F., but fell again to subnormal, rising again at 8 p.m. to 99.2°.

27. II. Temperature subnormal and remained normal or subnormal afterwards until 2. III. Patient allowed up on 1. III.

*Remarks.* The incubation period is about five days. The chart is perhaps the most typical of the series. It shows a distinct diphasic variation with maximum points of 104° F. and over, and a distinct remittent period.

The pulse was absolutely characteristic. It rose slightly with the first rise of temperature but thereafter fell and remained normal during the subsequent pyrexia. The occurrence of a temperature of nearly 105° F. with a pulse rate of 80 needs no further comment.

The extraordinary absence of symptoms was most impressive and is a striking instance of a pathological process affecting the thermo-regulatory system without obviously affecting any other.

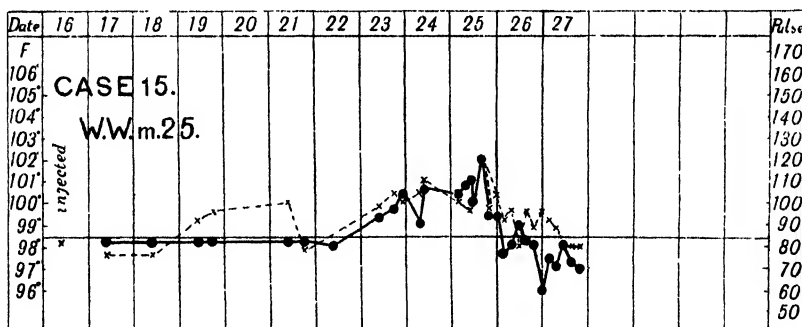
*Experimental material.* Although blood was twice taken with a view of using it for further experiments, the absence of volunteers prevented us making any use of the material.

Subcutaneous injection of 2 c.c. of undiluted serum filtrate from Case 10.

The blood was taken 67 hours after the onset. Virus kept outside the body 22 hours. *Result positive.*

**Case 15, W. W., m., 25 years.** On 16. II. 17, at 10 a.m., this case was injected subcutaneously with 2 c.c. of a "serum filtrate" from Case 10. The blood was taken from Case 10 on 15. II. at noon (67 hours after the onset). The temperature was normal until 23. II.

23. II. The patient became ill with typical and fairly severe symptoms. Malaise, bad headache and body pains were present. He continued to work. His temperature rose from 99.3° F. in the morning to 100.4° at 10 p.m.



24. II. Temperature at 7.30 a.m. 99° F. and at 9 a.m. 100.6°; it was not taken later that day. He was still going about his work but felt very sick. General pains were marked. There was pain in the eyes. He had a distinct

red mottled punctiform rash on the back, chest and buttocks, but not on the legs and arms.

25. II. Temperature rose to 102° F., but fell rapidly during the night. This day he remained in bed and felt very sick. Malaise and general pains were severe.

26. II. Maximum temperature 99° F.

27. II. Temperature was subnormal. He felt better and resumed ordinary duties. No further notes were taken but the rash was visible for several days and the patient complained of tiredness.

*Remarks.* The incubation period was seven days. This case was absolutely typical in symptoms and rash. The temperature variation is apparently monophasic. The pulse is not typical but it should be noted that this case had a tendency to a high pulse and was of a distinctly nervous temperament.

The blood from this case injected into Case 20 gave a *doubtful result*.

*Experimental material.* Blood was taken on 24. II. (24 hours after the onset), and 1.2 c.c. of the serum and corpuscles were injected into Case 20 on 27. II.

15 days later, Case 20 became febrile. His illness was most atypical, and cannot be considered definitely as dengue, especially as an attempt at further passage failed.

Subcutaneous injection of 1 c.c. of serum, containing corpuscles, taken from Case 10 on the eighth day of illness (about 190 hours after the onset).

The virus was kept outside the body at most a few hours. *Result negative*.

**Case 16, A. A., m., ? age.** On 20. II. 17, blood was taken from Case 10 and allowed to clot, and 1 c.c. of the serum was injected into this case at 5 p.m. The blood was taken eight days (190 hours) after the onset of Case 10. The virus was kept outside the body at most a few hours.

The chart was taken twice daily until 27. II., and thereafter every four hours until 13. III., and showed nothing to indicate any reaction to the injection.

*Remarks.* A *negative* result was obtained with serum from blood taken from Case 10 about 190 hours after the onset.

Subcutaneous injection of about 2 c.c. of blood filtrate from Case 11. The blood was taken 47 hours after the onset. Virus kept outside the body two days. *Result positive*.

**Case 17, N. K., f., 28 years.** On 14. II. 17, this patient received about 2 c.c. of a blood filtrate from Case 11 (*q.v.*). This had been prepared from blood drawn on 12. II. at 3 p.m. (47 hours after the onset of Case 11).

We have not a detailed history of this case as the circumstances of the volunteer did not permit us to take a chart, and when she became ill it was difficult to observe her frequently. The temperature was taken once daily



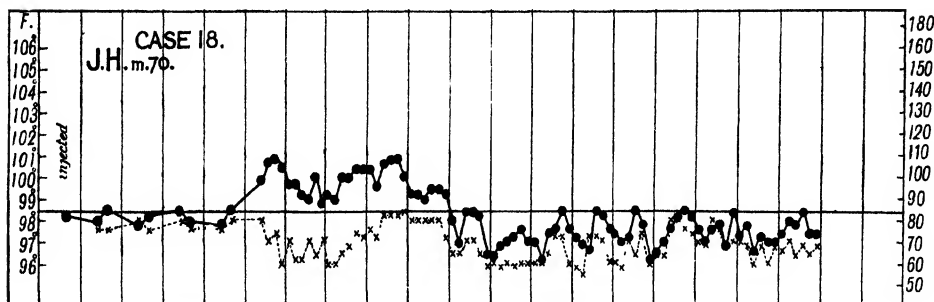
until 21. II. and was always normal and the pulse between 76 and 86. It was impossible to fix the onset as the patient did not say anything about feeling ill until we observed the rash, and then the history was not definite as to dates. But on 26. II. we observed that she had a very distinct rash, and on taking her temperature on the evening of that day it was found to be 100·3° F., with a pulse of 100, and the patient had the typical appearance of a dengue patient with pains, headache, lassitude, etc., and showed a bright typical rash on the face, forearms, chest and back (further examination was not made). The girl was obviously ill for some days after this, but refused to go to bed and was extremely anxious that her employers should not know that she had dengue, so further investigation was not pressed. There was no doubt that her case was very typical, the rash being one of the best of the series.

*Remarks.* Typical moderately severe dengue with well-marked rash.

Subcutaneous injection of 2·6 c.c. of "washings" from Case 11. The virus was taken from Case 11 47 hours after the onset, and was kept outside the body 48 hours.

**Case 18, J. F. H., m., 70 years.** On 14. II. 17, at 3 p.m., the case was injected subcutaneously with 2·6 c.c. of "washings" from Case 11 (*q.v.*). The blood was drawn from Case 11 on 12. II. at 3 p.m., 47 hours after the onset. The virus was kept outside the body 48 hours.

18. II. The patient complained of severe headache and pain in the chest on the afternoon of this date, but the temperature was normal.



19. II. At 10 a.m. the temperature was 99·8° F. and the pulse 80. Face flushed, eyes injected, tongue coated. There was severe headache. There was no rash but the back was erythematous. At 6 p.m. the temperature was 100·8° F. and the pulse 74. The patient was sleepless at night.

20. II. Headache still severe. Aspirin was given. There was no rash. The temperature was lower during the day (99·6° F.), but at 6 p.m. it rose to 100°, falling at midnight to 98·8°. He had a sleepless night.

21. II. There was nausea, pain in the chest, and headache requiring

aspirin. There was no rash. In the morning the temperature was 99° F., but in the evening rose to 100·4°.

22. II. Headache better. He had slept well during the night. Temperature reached 100·8° F., but fell during the night. Pulse for the first time rose somewhat, reaching 84 at 10 p.m.

23. II. He had still slight headache and anorexia, but felt better. Temperature not above 99·4° F. There was still no rash.

24. II. He complained of weakness and anorexia. The tongue was coated. Temperature normal or subnormal on this date and subsequently.

*Remarks.* The incubation period was between 4 and 5 days (about 4 days 19 hours to the first definite pyrexia). This is shorter than the usual incubation period. The temperature variation was diphasic with high points on 19 and 22. II.

The pulse was relatively slow throughout the pyrexial period. It was especially slow during the first phase of the fever.

Subcutaneous injection of 2·3 c.c. of dilute serum filtrate from Case 11. The blood was taken from Case 11, 115 hours after the onset, and was kept outside the body 70 hours. *Result negative.*

**Case 19, T. B., m., 55 years.** On 18. II. 17, at 10 a.m., Case 19 received subcutaneously 2·3 c.c. of a (1 to 1) diluted filtrate from the serum of Case 11 (*q.v.*). The blood was drawn from this case on 15. II. at about noon, about 115 hours after the onset. The virus, if present, would have been outside the body for 70 hours.

The temperature and pulse were taken twice daily for ten days, then every four hours for 13 days. The temperature reached 98·8° F. on 21. II., but thereafter was normal or subnormal.

*Remarks.* The late stage, at which the blood was taken from Case 11, by itself is enough to explain the *negative* result.

Subcutaneous injection of 1·2 c.c. of untreated blood from Case 15, taken 24 hours after the onset, and kept outside the body about 72 hours. *Result doubtful.*

**Case 20, C. L., m., 70 years.** On 27. II. 17, at 7 p.m., the case was injected subcutaneously with 1·2 c.c. of blood from Case 15, taken on 24. II. (about 24 hours after the onset). The virus was kept outside the body about 72 hours.

The temperature and pulse were taken twice daily for five days and then every four hours for three weeks. There was a rise to 99·2° F. on 5. III., about six days after the injection, but thereafter the temperature was practically normal. On 13. III. the temperature reached 99°, and on 14. III., 15 days after the injection, it rose to 100·2°, then fell rapidly, rose next day to 99·8°, fell again, and rose next day to 100°, falling rapidly again. The following day the maximum was 99°, and thereafter the chart was normal until 25. III., when the patient was discharged.

*Symptoms.* On 6. III. he developed a mild attack of herpes. On 7. III., although the temperature was only 98.8° F. at its maximum, there was some flushing of the face and back, and slight coating of the tongue. After this until 14. III. nothing was noted. Examined on this date the patient stated that he became shivery the previous evening at 7 p.m. He had some slight headache, some injection of the eyes, no coryza and slight cough without expectoration. On 15. III. there was an erythematous flushing of the back, no rash and a slight cough. On 16. III. he complained of nausea but otherwise remained well. On 17. III. he stated that he sweated every night. The tongue was slightly coated. Nothing else was noted after this.

There was relative bradycardia with the pyrexia on 14, 15 and 16. III., but no absolute bradycardia.

*Remarks.* The case cannot be definitely regarded as dengue, nor can it be stated that it was not.

In view of the failure to transmit infection from this case to Case 21, and the atypical nature and long incubation period, it is best to regard the case as *doubtful*.

*Experimental material.* Blood, taken from this case on 14. III., on the second (?) day of illness but the fifteenth after inoculation, failed to convey infection to Case 21.

Subcutaneous injection of 2 c.c. of untreated blood from Case 20, taken on the second (?) day of illness, and kept outside the body about 48 hours.

*Result negative.*

**Case 21, H. McD., m., 42 years.** On 16. III. 17, at 4.30 p.m., this patient was injected with 2 c.c. of blood taken on 14. III. from Case 20. He had thereafter no symptoms of dengue and no pyrexia, although observed daily for several weeks.

Subcutaneous injection of 2 c.c. of serum and corpuscles from a guinea-pig injected 7½ days before with blood from Case 10, taken 22 hours after the onset.

**Case 22, P. W. P., m., 71 years.** The details of this case are found under Part III, Animal Experiments (bottom of p. 233).

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## TWO INSTANCES OF HUMAN SERA SHOWING ABNORMAL ANTI-COMPLEMENTARY POWER.

BY E. L. KENNAWAY, M.D., D.SC., AND CAPT. A. M. WRIGHT, N.Z.M.C.

(*From the Bland-Sutton Institute of Pathology, Middlesex Hospital, and V.D. Sect., No. 3 New Zealand General Hospital.*)

IN the great majority of cases human sera which have been heated at 55–57° C. for thirty minutes, as is the usual practice preparatory to the Wassermann test, exhibit only very slight anti-complementary properties. That is to say, complete haemolysis of the test corpuscles (0.5 c.c. 3 per cent. suspension of ox or sheep red cells sensitised with five doses of immune body from the rabbit) usually occurs when these are added to a mixture of the amount of heated serum commonly employed (0.025 to 0.05 c.c. in 0.5 c.c. saline) along with 1½ to 2 doses of guinea-pig's complement previously incubated for 1½ hours at 37° C., the dose of complement being the amount which causes complete lysis when incubated with saline in the absence of the human serum. Scattered references to unusually anti-complementary sera occur in the literature, thus Thomsen and Bjarnhjedinsson recorded that the sera of lepers possessed very considerable anti-complementary action and this has been confirmed by Mathis and Beaujean among others. But excluding special treatment of sera, *e.g.* prolonged keeping as found by Browning and Mackenzie and confirmed by Zinsser and Johnson, or heating at higher temperatures, pronounced anti-complementary action is very rare.

In this paper we wish to draw attention to two instances of sera showing considerable anti-complementary power; in neither of these could any certain explanation of this abnormality be found either in the condition of the patient or in the mode of treatment of the serum. These are the only cases of the kind which we have observed in the performance of many thousands of Wassermann tests. The practical importance of this anti-complementary action is obvious, since such sera would inevitably be regarded as giving a positive Wassermann reaction if no control test of the serum alone were made.

The sera were inactivated as usual by heating for half-an-hour at 56° C. The serum control tubes contained 0.5 c.c. saline, 0.05 c.c. patient's serum, and amounts of complement as shown in the tables. The antigen employed in the Wassermann tests was an alcoholic extract of human heart plus cholesterol. The phenomenon was found to be independent of the species of red corpuscles employed, since the examination of Case I was carried out with those of the sheep, and that of Case II with those of the ox. The sera

were preserved after the first examination by freezing at from  $-10^{\circ}$  to  $-20^{\circ}$  C. All the results quoted below are based upon repeated examinations of which single examples only are given in the tables.

*Case I.* Five samples of this serum (A, B, C, D and E) were examined.

The patient contracted trench fever in June, 1918; on August 3rd of that year he received a gun shot wound in the right forearm which caused considerable injury to the flexor muscles and tendons; the wound was dirty when the patient came under treatment; the damaged portions were then excised. A week later severe haemorrhage from the wound occurred and 1000 c.c. of blood of Group II was given by transfusion.

On November 14th excision of the scar and secondary suture were carried out; sample A was taken before the anaesthesia, and sample B twelve hours later, the temperature was at this time within normal limits. After the operation the arm became oedematous and showed lymphangitis, and five days later the patient had a febrile attack, the temperature rising to  $105^{\circ}$  F. Sample C was obtained on November 29th, when the temperature had been normal for a week. Sample D was taken on December 19th, and E at the beginning of March, 1919. The state of the serum was thus observed over a period of four months. The patient presented no clinical evidence of syphilis.

The estimations of the full anti-complementary power of the serum are shown in Table I, and the results of the Wassermann tests carried out in the ordinary way in Table II.

Table I. Case I. *Anti-complementary Action of Serum.*

Date when sample taken	Complement, No. of M.H.D. ...	1.5	3	4.5	6	7.5
16. xi. 18	Sample A ... lysis	none	trace	marked	very marked	almost complete
	Sample B ... „	none	trace	distinct	very marked	almost complete
29. xi. 18	Sample C ... „	none	faint trace	trace	very marked	just complete
Complement, No. of M.H.D. ...						
19. xii. 18	Sample D ... lysis	1.3	2.6	4	5.2	—
		none	trace	just complete	complete	—
Complement, No. of M.H.D. ...						
1. iii. 19	Sample E ... lysis	2	—	—	—	—
	Samples A, B and C mixed and kept frozen since Nov. 1918; re-examined 3. iii. 19	complete	—	—	—	—
		complete	—	—	—	—

(1) Table I shows that samples A, B and C (Nov. 16th and 29th) showed almost identical anti-complementary action, lysis being in each case not quite complete in the presence of 7.5 M.H.D. of complement; the anaesthetic administered between the withdrawal of samples A and B had therefore no influence upon the condition. All other sera tested with the same batch of complement showed, as is almost invariably the case, complete or almost complete lysis with 1.5 or 2 M.H.D.; the inhibitory action of the serum in question must therefore have been six or seven times greater than normal.

Sample D, taken three weeks after sample C, showed just complete lysis with 4 M.H.D.; the anti-complementary power had therefore undergone diminution, though it was still distinctly abnormal. In sample E, taken two months after D, the normal condition was found to be re-established, lysis being complete with 2 M.H.D. The observations show, therefore, the decline and disappearance of the inhibitory character of the serum in the course of four months. These results obtained by one of us were practically duplicated by the other working independently at another laboratory. One other point may be mentioned: after the experiments carried out in November, 1918, the residue of the heated samples A, B and C were mixed and kept frozen at  $-10^{\circ}$  to  $-20^{\circ}$  C. until the following March, and then tested in the same way as before: the inhibition of lysis was found to have disappeared completely (Table I). This property must therefore be due to some quite unstable factor, since freezing is very effectual in preserving many of the properties of serum.

(2) The results of the Wassermann tests carried out in the usual manner with these sera are given in Table II; a comparison of these with the data given in Table I shows that the amounts of complement fixed in the presence of antigen (Table II) are in each of the five tests practically identical with

Table II. Case I. *Wassermann Tests.*

				Serum control
Complement, No. of M.H.D.	1.5	3	4.5	1.5
Sample A ... lysis	none	trace	distinct	faint trace
Sample B ... „	none	trace	distinct	faint trace
Negative control serum „	almost complete	complete	—	complete
Antigen control ... „	distinct	complete	—	—
Complement, No. of M.H.D.	1.5	3	4.5	1.5
Sample C ... lysis	none	faint trace	trace	none
Negative control serum „	complete	—	—	complete
Antigen control ... „	almost complete	complete	—	—
Complement, No. of M.H.D.	1.5	3	4.5	1.5
Sample D ... lysis	faint trace	marked	complete	faint trace
Negative control serum „	complete	—	—	complete
Antigen control ... „	complete	—	—	—
Complement, No. of M.H.D.	2	4	—	2
Sample E ... lysis	complete	—	—	complete
Negative control serum „	just complete	complete	—	complete
Antigen control ... „	complete	—	—	—

those inhibited by the sera alone (Table I). For instance, sample C gave a trace of lysis with 4.5 M.H.D. of complement both in the presence and in the absence of antigen. The Wassermann reaction was, therefore, negative; as was mentioned above, the patient showed no clinical evidence of syphilis. The results with the negative control serum included in Table II show that sera such as samples A, B, C and D would inevitably be regarded as giving a positive Wassermann reaction if no control observations were made with

the serum alone; whereas the employment of proper serum controls, such as are recorded in the last column of the table, causes the abnormality to be at once detected.

The apparent positive Wassermann reaction diminished *pari passu* with the loss of anti-complementary power, until in the last sample (E) the behaviour of the serum is seen to be practically identical with that of the negative control. Incidentally the results in Table II show that the administration of the anaesthetic (Nov. 16, samples A and B) had no influence upon the strength of the Wassermann reaction. This is of interest in view of statements that an anaesthetic may cause the serum to react positively.

*Case II.* Female, aged 56. As regards the clinical history of the case, we have been able to learn no more than that the patient showed "mental symptoms." The serum (sample A, Table III) showed somewhat less inhibitory power than did the first samples in Case I, lysis being complete with 5 M.H.D.

Table III.

Case II. (1) *Anti-complementary Action of Serum.*

Date	Complement, No. of M.H.D.	2	3	5
3. iii. 19	Sample A ... lysis	none	distinct	complete
2. iv. 19	Sample B ... „	complete	—	—

(2) *Wassermann Test.*

	Complement, No. of M.H.D.	1.5	3	4.5	Serum control 1.5
3. iii. 19	Sample A ... lysis	none	none	none	faint trace
	Negative control serum „	complete	—	—	complete
	Antigen control „	complete	—	—	—
	Complement, No. of M.H.D.	2	4	6	2
	Sample B ... lysis	none	none	none	complete
2. iv. 19	Negative control serum „	complete	—	—	complete
	Antigen control „	complete	—	—	—

of complement. In contrast to Case I, the Wassermann reaction was found to be positive, no lysis occurring in the presence of antigen with 4.5 M.H.D., whereas in the absence of antigen lysis would no doubt have been almost if not quite complete with this amount. All other sera tested on this occasion showed complete or practically complete lysis with 2 M.H.D. The serum was somewhat deeply tinted with haemoglobin, but we have examined hundreds of such sera from partially lysed bloods without encountering any other instances of anti-complementary action.

A month later a second sample (B), free from haemoglobin, was examined. In the meantime the patient had received anti-syphilitic treatment ("914" and calomel), an injection having been given a week before the withdrawal of the blood. The Wassermann reaction was found to be as strongly positive as before (no lysis with 6 M.H.D.), but the abnormal anti-complementary power had disappeared, lysis being complete in the serum control tube with 2 M.H.D.

The second case thus resembles the first in that the anti-complementary property was transient only, disappearing in the course of from one to three months. That this fixation of complement is independent of that which is the basis of the Wassermann reaction is shown by the facts that (1) the Wassermann was in Case I negative and in Case II positive, and (2) in Case II the anti-complementary power disappeared while the Wassermann remained positive. The two cases do not present any common feature which would suggest the cause of the abnormality in question.

#### SUMMARY.

Two instances are described of sera showing abnormal anti-complementary power. The amount of complement fixed by the serum was in the one case about six times, in the other about four times, greater than is normal. Examination of subsequent specimens from the patients showed that this inhibitory character was transient only. No feature common to the two cases was found to which the abnormality could be attributed. Attention is drawn to the rarity of this condition, but it is of practical importance in that such sera would be regarded as giving a positive Wassermann reaction if their behaviour in the absence of antigen were not observed.

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## OBSERVATIONS ON THE CULTIVATION OF TYPHOID AND PARATYPHOID BACILLI FROM THE STOOLS WITH SPECIAL REFERENCE TO THE BRILLIANT GREEN ENRICHMENT METHOD.

BY CAPTAIN J. W. MCLEOD, R.A.M.C., 8th MOBILE LABORATORY.

THE question of standardising bacteriological methods is at present under discussion and, although it is most undesirable that any one method should be forced on all bacteriologists, it is certainly reasonable that any method which has given excellent results in the hands of a number of independent workers should have a strong recommendation.

Browning, Gilmour, and McKie (1913) described a method for isolating Typhoid bacilli from the faeces by means of an incubation in dilute solutions of Brilliant Green prior to plating, and the value of the method has been investigated in many laboratories in the course of this war. Glynn and his collaborators (1917) summarised the published work dealing with this subject which had appeared up to the date of their report, and they expressed the general conclusion that "the available evidence indicates that the advantage of Brilliant Green, certainly of Browning's simplified technique, is not sufficiently established to justify its being recommended as an additional routine method in laboratories where enterics are examined, especially having regard to the slight extra labour and cost." In as much as the method has been, in my experience, one of the most valuable modifications of bacteriological technique which have been introduced and since it would seem to me to be a misfortune if the opinion expressed in the report quoted should deter any who are unfamiliar with the method from giving it a trial, I have not hesitated to publish the results which follow, although the conclusions may seem to some to be already well established.

The observations relate to the work of a Mobile Laboratory during the last four years. The opportunities for observing cases of infection of the Typhoid group have been the following: (i) a series of paratyphoid infections in the troops withdrawn from the Ypres sector to the area north of Albert in the summer of 1915; (ii) a small epidemic of typhoid amongst the civilians east of Doullens at the same period; (iii) a small epidemic of paratyphoid infection amongst the troops of a division which returned from Egypt in the early part of 1916; (iv) very occasional cases of typhoid or paratyphoid infections occurring amongst the troops or civilians in the area before and behind Cassell in 1917 and 1918; (v) a considerable outbreak of typhoid

infections amongst German civilians at Euskirchen in the end of 1918 and the beginning of 1919.

The observations made in the first two groups were mostly limited to blood culture, but in a considerable proportion of cases in the other groups a careful comparison between the value of direct plating and of Brilliant Green enrichment was made. In 1916 one tube only of Brilliant Green was used, a 1/250,000 dilution, and the method compared was that described by Ledingham and Arkwright (1912) with the modification that only one, not two or three plates were used for each specimen. In 1917, 1918 and 1919 1/250,000 and 1/500,000 dilutions of the dye were employed and the comparison was made with a literally direct method, *i.e.* the plate was inoculated with a small portion of faeces and spread immediately. The results of all investigations up to the end of 1918 in which the direct and Brilliant Green methods were compared are set down in Table I. These observations refer chiefly to paratyphoid infections and it is seen that out of the 16 results obtained 15 were

Table I.

Date	Name	Brilliant Green	Result
			Direct
30-4-16	Driver S.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
30-4-16	Pte. C.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
21-5-16	Pte. W.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
30-5-16	Pte. Lambert (1)	Paratyphosus A, nearly pure culture	Negative
	" (2)	Paratyphosus A, one colony	Negative
	" (3)	Paratyphosus A, nearly pure culture	Negative
12-6-16	Pte. S.	Paratyphosus B, colonies	Paratyphosus B, colonies
5-7-16	Pte. A.	Paratyphosus B, numerous colonies	Paratyphosus B, scanty colonies
24-8-16	Pte. F.	(1) Paratyphosus B, colonies	Negative
	"	(2) Paratyphosus B, colonies	Negative
*15-7-17	Pte. S.	Negative	<i>B. typhosus</i>
26-7-17	Pte. S.	Paratyphosus B	Negative
16-9-17	Pte. D.	Paratyphosus B, about 100 colonies	Paratyphosus B, about 20 colonies
29-11-17	Rfm. D.	Paratyphosus B	Negative
18-7-18	Civilian	Paratyphosus B, isolated	Negative
29-9-18	Civilian	<i>B. typhosus</i>	Negative

\* A tube of Brilliant Green solution which had been standing for some time and become discoloured was used on this occasion.

positive by the Brilliant Green method and only 7 out of 16 were positive by the direct method.

It was the case of Lambert (Table I) in this group which brought out the value of the method in the most striking way. An investigation had been required of all possible carriers in this man's regiment on account of a rather severe outbreak of paratyphoid infection. After investigating without success the stools and urines of 60 other men, I obtained an almost pure culture of

Paratyphoid A from the Brilliant Green tube of this man's stool but nothing except *B. coli* and few colonies of coarse non-lactose fermenters from the direct plate. The examination was repeated twice with the results cited in Table I confirming the original finding.

The man's history was that he had had a sharp attack of diarrhoea in Egypt, and had suffered from irregularity of the bowels ever since. The regimental epidemic had been a mixed one, "A" and "B" infections occurring simultaneously, but the latter predominating. No more cases of "A" infection occurred after the detection of Lambert. A continued investigation for a "B" carrier was unsuccessful; the "B" infections gradually died out, however, and were possibly all due to case to case contact.

In view of the above result I find it impossible to accept the opinion expressed by Glynn and his collaborators, as quoted above. Extra trouble and cost would have been involved by omitting the use of the Brilliant Green method in this case, and at least one month's fruitless work would have had to be carried out and possibly a continued series of paratyphoid "A" infections would have occurred in the regiment.

The claim, however, originally made by Browning, Gilmour and McKie for the Brilliant Green method was not only that it facilitated the isolation of paratyphoid bacilli but that of typhoid bacilli. Most of those who from their practical experience of the method in this war have written in its favour, only recommend it, however, in respect of the isolation of the paratyphoid bacilli (Stokes and Clark, 1916; Fletcher, 1917).

Table II.

Name		Stool		Urine	
		Direct	Brilliant Green	Direct	Brilliant Green
Sophie U. ...	...	Abundant Paratyph. B	Abundant Paratyph. B	Abundant Paratyph. B	Abundant Paratyph. B
Gertde. M. ...	...	Negative	Scanty colonies <i>B. typhosus</i>	Negative	Negative
Christ. D. ...	...	Approx. 10 col. of <i>B. coli</i> to 1 of <i>B. typhosus</i>	Approx. 1 col. of <i>B. coli</i> to 4 of <i>B. typhosus</i>	Negative	Negative
Schwester A.	...	One or two col. <i>B. typhosus</i> , <i>B. coli</i> abundant	Approx. 5 col. <i>B. typhosus</i> to 1 col. <i>B. coli</i>	Negative	A few col. <i>B. typhosus</i>
Frau S. ...	...	Negative	A few col. <i>B. typhosus</i>	Negative	Negative
Jacob S. ...	...	Negative	Paratyph. B	Negative	Negative
Heinrich C. ...	...	Negative	<i>B. typhosus</i>	Negative	Negative
Helene P. ...	...	<i>B. typhosus</i>	Negative	—	—
Frau S. (2nd exp.) ...	...	Negative	<i>B. typhosus</i>	—	—
Frau K. ...	...	Negative	<i>B. typhosus</i>	—	—
Frau W. ...	...	<i>B. typhosus</i>	<i>B. typhosus</i>	—	—
Aug. U. ...	...	<i>B. typhosus</i>	<i>B. typhosus</i>	—	—
Frau K. (2nd exp.) ...	...	Negative	<i>B. typhosus</i>	—	—
Baxter, 1st spec. ...	...	Negative	<i>B. typhosus</i>	—	—
„ 2nd spec. ...	...	<i>B. typhosus</i> col. scanty	<i>B. typhosus</i> col. numerous	—	—

The occurrence of the civilian epidemic already mentioned in the early part of 1919 at Euskirchen, a small town in the British area of occupation west of the Rhine, afforded a good opportunity of retesting the value of the Brilliant Green enrichment method in cases of infection with *B. typhosus*.

The results obtained are given in Table II.

Excluding the two paratyphoid infections, there were 14 specimens derived from 11 different cases, 13 of stool and one of urine, in which a positive result was obtained. In 13 of these the result was positive by the Brilliant Green method, whereas in 6 only by the direct method. In my experience therefore the original claim for the efficacy of the method in isolating *B. typhosus* from the stool is fully vindicated.

#### *Technique.*

A few words about the technique appear relevant since it is probably the source of varying results amongst different workers. The dilutions of Brilliant Green in peptone water have been made by adding with a sterile graduated pipette the requisite quantities of  $\frac{1}{10000}$  solution of Brilliant Green to sterile tubes of peptone water each containing 10 c.c. Solutions of Brilliant Green in peptone water were not autoclaved although I have no proof that this has any deleterious effect. The original technique was persisted in as it had given good results. The Brilliant Green tubes were always inoculated copiously, much more material being transplanted than could be adequately spread directly on several plates.

The peptone water used was capable of yielding a rapid and copious growth of *B. typhosus*, and suitable Brilliant Green was employed. These two factors are important. If peptone water is neutralised by a fixed addition of alkali and not titrated, it may easily happen that in frequent moving a laboratory will strike some water supply of an unusual grade of alkalinity or acidity, which if used to prepare peptone water according to formula will yield a product incapable of promoting a rapid growth of *B. typhosus*.

A number of bottles of "Brilliant Green" crystals have been issued through the Army Depots of Medical Supplies which have neither had the crystalline appearance nor the antiseptic properties of Brilliant Green. An old bottle of Grüber's preparation has been used throughout in these investigations, and it does not seem to me that any criticism of this method is pertinent unless the work is carried out with Grüber's Brilliant Green or with a specimen which has been proved equal to it in parallel experiment.

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# THE BACTERIAL CONTENT OF THE AIR IN ARMY SLEEPING HUTS, WITH ESPECIAL REFERENCE TO THE MENINGOCOCCUS.

BY A. J. EAGLETON, M.D.

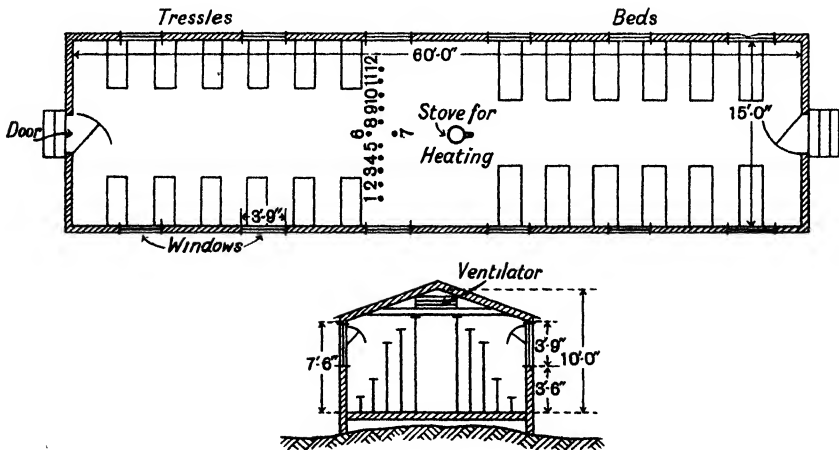
(Formerly Officer i/c Laboratory, Wylde Area.)

(With 1 Diagram.)

THE following experiments were carried out in October and November, 1917. They have not been published hitherto as it was hoped that they might be extended and revised.

The onset of the influenza epidemic, the armistice and the resulting dispersal of troops prevented this, and in so far as some of the conclusions arrived at seem of no little importance, it has been thought advisable to publish the results of the work done, incomplete though it is.

The first series of experiments I-VIII deals with the number of organisms found on plates exposed during the night in men's sleeping huts, Barrack Design\* 230/14. The same hut was always used, and the number of men in the hut was always 22.



Men's Sleeping Hut: Barrack Design 230/14.

This diagram shows the ground plan and vertical section of the type of hut in which the experiments were conducted.

The "beds" used were either ward beds or plank trestles, but no appreciable difference in the bacterial content of the hut could be detected whichever type was employed. It will be noticed that there was a ventilator in the roof extending the entire length of the hut and that there were six windows on each side, the upper half of which could be opened. There was no ground ventilation but the huts were floored with planks and raised above the grass on wooden supports.

### *Experiment I.*

Plates exposed 10.30 p.m., 25.10.17.

Plates removed to incubator 6.0 a.m., 26.10.17.

Temperature in hut: Maximum 58° F., Minimum 48° F.

Plates	Level	Colonies
1, 12	1 foot above ground	All plates showed innumerable colonies; no mould or other gross contamination.
2, 11	3    "    "	
3, 10	5    "    "	
4, 9	6    "    "	
5, 8	7    "    "	
6, 7	Ground level	

### *Experiment II.*

Plates exposed 10.30 p.m., 28.10.17.

Plates removed 6.0 a.m., 29.10.17.

Temperature in hut: Maximum 56° F., Minimum 44° F.

Plates	Level	Colonies
1, 2	8 feet above floor	All plates showed innumerable colonies; Nos. 5 and 9 were overgrown with mould.
3, 4	7.5 "    "    (inverted)	
5	7    "    "	
6	6    "    "	
7	5    "    "	
8	4    "    "	
9	3    "    "	
10	2    "    "	
11	1    "    "	
12	Floor level	

### *Experiment III.*

A comparison of the effect of beds and trestles. The height of the men's heads above the floor was 2 feet 3 inches and 1 foot 3 inches in the two groups.

Plates	Level	Colonies
2, 6, 12, 14	Ground level	All plates in both sets showed innumerable colonies; none overgrown.
4	2 feet	
5	1½ "	
1	2½ "	
13	3    "	
9	3½ "	
11	4    "	
7	4½ "	
8	5    "	
15	6    "	

Two sets of plates used, one between beds and the other between trestles.

*Meningococcus**Experiment IV.*

An exact repetition of Exp. III with the same result.

*Experiments V and VI.*

Two identical experiments to show the number of bacteria in the air in spaces between beds and trestles at higher levels.

Plates exposed 19.10.17, midnight.

Plates removed 20.10.17, 6.0 a.m.

Temperature: Maximum 58° F., Minimum 40° F.

Plates	Level	Colonies
5	7 feet	In both sets innumerable colonies; none overgrown.
4	6½ "	
1	6 "	
6	5 "	
3	4 "	
2	3 "	

Two sets as in Experiments III and IV.

*Experiment VII.*

Plates placed as high as possible in intervals between beds.

Plates exposed 10.30 p.m., 20.10.17.

Plates removed 6.0 a.m., 21.10.17.

Temperature: Maximum 50° F. (?), Minimum 49° F.

Plates	Level	Colonies
1, 2, 3, 4	8½ feet	All plates showed innumerable colonies, except 5 and 7 (both inverted). 5, 250 colonies 7, 360 "
7, 8, inverted	7½ "	
9, 10	7 "	
6, 5, inverted	6½ "	

*Experiment VIII.*

Same as VII, only trestles instead of beds.

Plates exposed 21.10.17, midnight.

Plates removed 6.0 a.m., 22.10.17.

Temperature: Maximum 56° F., Minimum 48° F.

All plates showed innumerable colonies.

Experiments I–VIII show therefore that in the type of army hut investigated the bacterial content of the air during sleeping hours is very high and that the air throughout the hut is uniformly infected.

*Experiment IX.*

A larger type of hut used. Plates placed all on ground level.

Plates exposed 10.10.17, 10.0 p.m.

Plates removed 11.10.17, 6.0 a.m.

Temperature: Maximum 55° F., Minimum 45° F.

Plates	Position	Colonies
2, 3, 5, 6	Between trestles	Uncountable
1	In centre of room	48
4	" "	30

### *Experiment X.*

Hospital ward. Space between beds 2 feet. Plates placed between beds.

Plates exposed 14.10.17, 9.30 p.m.

Plates removed 15.10.17, 4.30 a.m.

Temperature: Maximum 54° F., Minimum 43° F.

Plates	Level	Colonies
1	Ground	152
2	"	164
3	"	Overgrown
4	"	"
5	1 foot	176
6	1 "	Overgrown
7	1½ feet	184
8	1½ "	Overgrown
9	2 "	166
10	2 "	Overgrown
11	2½ "	"
12	2½ "	127
13	3 "	101

It is seen that, although the bacterial content of the air is less than in the ordinary army hut, still the number of colonies obtained is very considerable both at ground level and at 3 feet.

### *Experiment XI.*

Comparison of condition in a half hut, type 230/14, when 5 feet was allowed between each bed, the men being carriers of meningococcus.

Plates exposed 13.10.17, 10.0 p.m.

Plates removed 14.10.17, 6.30 a.m.

Temperature: Maximum 38° F., Minimum 36° F.

Plates	Level	Colonies
10	Ground	540
11	"	496
12	"	450
13	"	450
1	1 foot	Overgrown
2	1 "	546
3	1½ feet	304
4	1½ "	? slightly overgrown
5	2 "	308
6	2 "	300
7	2½ "	300
8	2½ "	276
9	3 "	250

Meningococci were present in No. 4. In all cases where meningococci are stated to be present, they were proved by agglutination to be genuine strains.



*Experiment XII.*

To show the presence of meningococci in the air. Patients were all known carriers.

Plates exposed 11.10.17, 10.0 p.m.

Plates removed 12.10.17, 6.30 a.m.

Temperature: Maximum 53° F., Minimum 47° F.

*Plates.* Six exposed on ground level, in intervals between beds. All showed innumerable colonies. Meningococci present, but only one plate showed several colonies.

*Experiment XIII.*

More exact experiment on conveyance of meningococcus. Chronic carrier lying on trestle for 5 minutes, coughing periodically. Plates arranged on the ground at different distances from his mouth, which was 1 foot 9 inches above the floor. The room had been previously sprayed with formalin and the floor scrubbed and cresoled.

Plates	Distance from subject in feet	Meningococci
1, 2	6	overgrown
3, 4	7	"
5, 6	8	"
7, 8	9	"
9, 10	10	negative
11, 12	11	"
13, 14	12	overgrown
15, 16	13	positive
17, 18	14	overgrown

*Experiment XIV.*

Chronic carrier sleeping with face towards plates. Plates all on ground level. Same preparation of room as in Experiment XIII.

Plates exposed 12.10.17, 10.0 p.m.

Plates removed 13.10.17, 6.30 a.m.

Plates	Distance in feet	Meningococci	Colonies
1	3	? not proven	Uncountable
2	3	+	320
3	5	+	276
4	5	+	360
5	6	overgrown	
6	6	"	
7	7	"	
8	7	-	372
9	8	-	288
10	8	-	204
11	9	-	172
12	9	-	190
13	10	overgrown	
14	10	"	
15	11	-	160
16	11	-	140
17	12	overgrown	
18	12	-	146

*Experiment XV.*

Exact repetition of Experiment XIV.

Plates exposed 9.10.17, 10.0 p.m.

Plates removed 10.10.17, 6.30 a.m.

Plates 1, 3 and 4 showed meningococci, i.e. at distances of 3 and 5 feet.

*Experiment XVI.*

Same as preceding. Plates at different distances and levels from face of carrier.

Plates exposed 15.10.17, 10.0 p.m.

Plates removed 16.10.17, 6.30 a.m.

Temperature: Maximum 53° F., Minimum 50° F.

Plates	Level	Colonies	Meningococci
7 (1 foot from subject)	2½ feet	45	+
20     "     "	4     "	40	-
13     "     "	5     "	overgrown	
3     "     "	1½   "	"	
11     "     "	3½   "	44	+
6 (2 feet from subject)	6     "	overgrown	
2     "     "	1     "	30	-
15     "     "	5½   "	38	+
8     "     "	3     "	45	+
4 (3 feet from subject)	5     "	overgrown	
18     "     "	3½   "	54	+
14     "     "	3     "	overgrown	
12     "     "	5½   "	"	
17     "     "	1½   "	smashed	
16 (4 feet from subject)	2½   "	overgrown	
5     "     "	4½   "	"	
1     "     "	2     "	150	+
19 (5 feet from subject)	4     "	60	-
9     "     "	4½   "	overgrown	
21     "     "	2     "	56	-
22     "     "	1     "	56	-
10     "     "	6     "	overgrown	

## SUMMARY.

*Experiments I-VIII.*

I shows that under the conditions shown the plates were covered with innumerable colonies 7 feet above ground level to ground level. The beds and trestles were used to keep the hut always uniform.

II shows that 8 feet above ground, i.e. on top of the crossbeams, the same conditions obtain.

III, IV, V, and VI show that the same condition exists in the area between the beds or trestles. The beds make no difference, better or worse.

VII shows practically the same condition high up on the beam between the beds. The inverted plates naturally show less colonies, as the organisms mostly fall on to the plates.

VIII confirms the above.

There is only one conclusion to draw from these experiments, and that is that practically speaking there is no difference in the number of organisms on the ground floor and 8 feet above it, in the ordinary men's sleeping huts, Barrack Design 230/14.

*Experiments IX-XII* are shown for comparative reasons.

IX shows a better condition in a broader hut, although more men were sleeping in it.

X shows the superiority of the ward buildings. The beds were only 2 feet apart and chosen specially to show effect of overcrowding.

XI shows effect of bed space and low temperature. It also shows possibility of transfer of meningococci. The meningococci were proved by agglutination tests.

XII was a control on XI. The effect of the higher temperature is seen, also possibility of spread of meningococci.

#### *Experiments XIII-XVI.*

These are more exact experiments on distance to which meningococci can be carried. "Spraying capacity of patient."

XIII. By coughing it was carried 13 feet. Several like experiments were done, but owing to overgrowth by *subtilis*, etc., this was the only one where the organism could be proved by agglutination. Others showed suggestive colonies but they could not be isolated.

XIV. This showed that in ordinary sleep a carrier can spray to a distance of 5 feet.

XV confirms above, and from these two and several other negative experiments it seems justifiable to conclude that the "spraying capacity" of a carrier during ordinary sleep is 5 feet along the level or just below it.

XVI shows that meningococci are carried during sleep:

1 foot at a height of 2 ft 6 in.			
1	"	"	3 " 6 "
2	"	"	5 " 6 "
2	"	"	3 " --
3	"	"	3 " 6 "
4	"	"	2 " —
5	"	nothing above ground level	

so that we may say that in ordinary sleep the organism is not carried more than 5 feet, but in violent coughing it may be carried three times this distance.

The conclusions to be drawn from the above experiments, limited as they are, seem to be the following:

(1) In the ordinary infantry sleeping hut, there is a stagnant well of infected air in which the men sleep.

(2) This dead space extends up to 8 feet above the ground level.

(3) The wider the hut and the lower the temperature, the better the condition of the air.

(4) More adequate ventilation is needed; probably this could be obtained by ground ventilation.

(5) The meningococcus can be carried at night from a carrier to his neighbours unless the bed space is more than 5 feet.

(6) The spraying capacity of a carrier varies between 5 and 15 feet, but is 5 feet during ordinary sleep.

(7) The meningococcus is carried in the spray to a much shorter distance than many other organisms.

In conclusion, I have the pleasant task of thanking those who have helped me to carry out this piece of work. Colonel Morse, R.A.M.C., arranged for the use of the hut and the discipline of the subjects. Capt. Welsford, Senior Sanitary Officer, interested himself in the work and obtained for me the services of Lt. Hasnip who provided the diagrams of the huts. Finally, Colonel Mervyn Gordon criticised the earlier experiments and supplied suggestions for new work, which unfortunately I was unable to carry out.

# AN EXPERIMENTAL INVESTIGATION OF AN AUSTRALIAN EPIDEMIC OF ACUTE ENCEPHALO-MYELITIS<sup>1</sup>.

By J. BURTON CLELAND, M.D., AND A. W. CAMPBELL, M.D.

(*From the Microbiological Laboratory, Department of Public Health,  
New South Wales.*)

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<sup>1</sup> A full epidemiological, clinical and histological account of the disease may be read in the Annual Report of the Microbiological Laboratory of the Department of Public Health, Sydney, N.S.W., for the year 1917.

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## I. INTRODUCTION.

THIS investigation concerns an epidemic disease, which for convenience we have called "X disease," prevalent in certain parts of New South Wales during the late summer of the years 1917 and 1918.

The disease was at first thought to be "acute poliomyelitis"—the Heine-Medin disease. Increased observation, however, revealed important discrepancies; thus (1) it was confined to "outback" towns or districts remote from one another and from the metropolis (Sydney), and did not synchronise with any metropolitan epidemic of "acute poliomyelitis"; (2) it was extremely fatal (we have notes of 134 cases, of which no less than 94 died); (3) it attacked a number of adults (34 cases); (4) signs of intense cerebro-spinal irritation (convulsions, rigidity, increased reflex activity, mental confusion and pyrexia) dominated the clinical picture, paralysis, intercurrent or residual, being infrequent; and (5) the histological picture differed from that of "acute poliomyelitis," inasmuch as the changes were distributed throughout the central nervous system and did not fall with special intensity on the spinal cord.

Impressed by the peculiar features of the disease, we set in train the series of experiments herein recorded. The list comprises 62 experiments on monkeys, 52 on sheep, four on calves, one on a horse, and sundry experiments on dogs, kittens, rabbits, guinea-pigs and a fowl. Hence the research has been extensive. We may also say now that, while various side issues have been followed up with interesting results, the finding of chief importance is that the disease is communicable not only to the monkey, but to the sheep, the horse and the calf, that is, to animals which, so far as we are aware, have hitherto proved resistant to "acute poliomyelitis"—which is an additional discrepancy.

## II. INOCULATION, MATERIALS AND METHODS.

The usual procedure in obtaining and maintaining the virus for inoculation purposes was the following:

At the autopsy of the human being or animal dead from the disease, thin

slices of tissue were taken from the frontal, parietal, occipital and temporo-sphenoidal regions of the cerebrum, from the cerebellum, from the pons and medulla, and from the cervical, dorsal and lumbar areas of the spinal cord. These were then straightway put into 33 per cent. glycerine diluted either with water, or in the earlier cases with normal saline solution, in which they were kept in an ice chest until wanted; or the material was at once emulsified with sterile powdered quartz in a mortar with the diluent, and stored similarly after a proper milky emulsion had been made. To preserve material from one or two of the earlier human cases the strength of the glycerine solution was 50 per cent. In some cases the emulsion was made with normal saline solution only when it was proposed immediately to inoculate a test animal. When small blocks of tissue had been preserved in glycerine, they were emulsified in a mortar just before inoculation. Pasteur-Chamberland F. and Berkefeld filtrates of this primary emulsion of the brain or spinal cord were sometimes used, whilst in other cases, after light centrifuging, the supernatant fluid was treated with various sera.

Other materials used for inoculation comprised swabbings from the nasopharynx emulsified in 33 per cent. glycerine solution, a Pasteur-Chamberland F. filtrate of faeces from a human case, and an emulsion of fowl ticks.

Nearly all the inoculations were made intracerebrally. The usual procedure was to tie the animal out on a frame and to anaesthetise it, first with chloroform and then with ether; in some cases chloroform alone was used. After cleaning the scalp and removing the hair, a small lineal incision was made in the parietal area down to the bone. Then by means of a small trephine, a puncture was made through the skull by means of the pin in the centre of the trephine, the teeth of the trephine keeping the pin in position. It was found better to use a trephine than an awl or other instrument. As a rule the time when the pin of the trephine had pierced the cranial vault could be easily estimated by the teeth of the trephine beginning to give. Occasionally, when a little undue force was used, the disc of bone loosened by the trephine became somewhat depressed. In one or two of the earlier cases the trephine tablet was removed. This was apt to lead, when the disease developed, to a hernia cerebri. The puncture having been made and by means of a surgical needle proved to be through the bone, a moderate sized needle of a large 5 c.c. syringe was inserted deeply into the cerebral substance, and about 1 c.c. of the emulsion injected. At one time the injection was made with some force, and the needle end was moved in various directions, with the deliberate object of destroying a certain amount of brain tissue in the neighbourhood of the injection and so facilitating the "taking" of the virus. It frequently happened that on withdrawing the syringe fluid escaped along the needle track but in several cases wherein this occurred the animals nevertheless contracted the disease.

The animals appeared to recover perfectly from the operation, usually within a few hours, and next day, with few exceptions, showed no results of



the operative treatment, in spite of the fact that definite injury must have been done to the brain.

In some of the earlier cases not only was the emulsion injected into the brain substance, but a certain amount was injected subdurally as well.

Intraperitoneal and intrasciatic inoculations were made in the usual way. In two cases lumbar puncture was performed, once in a monkey and once in a sheep, and a few c.c. of normal serum were injected into the spinal canal. Then an incision was made in the thigh or neck of the animal and about 0.5 c.c. of the emulsion was injected intravenously. No results followed from any of these inoculations. In the case of the intravenous introductions, the failure was possibly due to the small amount of virus employed.

### III. DETAILS AS TO THE VIRUSES EMPLOYED.

1. *Estimated day of illness when the virus was obtained.* This varied from one to eight days; in most cases it was obtained on the third to the fifth day. In the three human cases, the patient had died on the third, second and fifth days of the disease respectively.

2. *Hours after death when the virus was removed from the body.* With the exception of the cases in which the virus was obtained from human beings, the post-mortem examinations were made immediately after death or within a few hours. The three human cases had been dead 5, 12 and 21 hours respectively.

3. *Period during which the virus was preserved in vitro.* In a number of the animal experiments this was only a few hours. The longest periods during which it was preserved in glycerine and retained its activity were seven days in one human case and fifteen days in a monkey case.

4. *Menstruum in which the virus was preserved.* When the virus was immediately injected into a further test animal, it was usually suspended in normal saline solution. In most of the other cases it was preserved in 33 per cent. glycerine solution, the dilution of the glycerine being sometimes with normal saline solution, sometimes with sterile water. The percentage of glycerine was usually 33, but in one instance it was 50.

### IV. THE DISEASE AS MANIFESTED IN MONKEYS (*MACACUS RHESUS*).

Twenty monkeys altogether were successfully inoculated with the virus as proved by full histological examination after death. Details of the individual animals will be found in the Appendices.

1. *The incubation period.* This varied from 5 to 23 days, the average of the 20 cases being 10.25 days, or eliminating the very long incubation period of 23 days, 9.5 days. In only one instance was the incubation period as long as 23 days; in one it was 17 days; in one, 15 days; in two, 12 days; in three, 11 days; in three, 10 days; in two, 9 days; in one, 8 days; in three, 7 days; in one, 6 days; and in two, 5 days.

2. *Length of illness.* This varied from  $2\frac{1}{2}$  to 11 days. In most instances the animals were killed *in extremis*. In several instances it is possible, more especially in the case of A. 110, Monkey 3925, that the animal might have survived a considerably longer period, or have recovered. In the majority of cases, however, the animal had reached an almost moribund state in from 3 to 6 days.

3. *The general course of the disease.* In half, at least, of the affected monkeys, the first sign of the onset of the disease was a peculiar anxious facial expression. This sign might precede by a day other manifestations, or might be accompanied by evidences of incoördination and by exaggerated or irregular muscular movements and increased reflex action. In most instances there was a gradual progression of this incoördination and exaggerated muscular movement, sometimes accompanied by convulsive muscular contortions, and occasionally by true convulsions, until eventually the animal lay prostrate on the bottom of the cage. In some instances paresis of a limb, accompanied by incoördination, was a recognisable and early manifestation, and in one case prostration was the first indication of a "take." In some cases consciousness was apparently not lost until just before the animal was killed or died. The dominant features of the disease were exaggerated muscular movements and intense incoördination, though a few individual monkeys were quieter, and showed less incoördination, and more paresis.

4. *The symptoms and signs manifested on the first day of illness.* In ten of the twenty monkeys an anxious or frightened expression was easily recognisable on the first day of illness, and as already indicated was frequently the first intimation that the virus had "taken." In eight cases the monkeys were described as being nervous or jumpy, or as walking gingerly; in eight also there was definite incoördination of muscular movement. These two classes comprised fourteen of the twenty monkeys. Some spasticity of the limbs was noticed in one monkey, and convulsions were recorded in one. In six monkeys there was definite paresis of a limb, and in one the animal was found prostrate.

Regrouping the above signs, it will be found that on the first day of illness fifteen of the twenty monkeys showed exaggerated movements, incoördination, spasticity, or convulsions, whilst only seven showed paresis and none paralysis.

5. *Later manifestations.* The most note-worthy feature of these cases, seen in nineteen of the twenty monkeys, was an incoördination of movement of a form apparently due more to exaggeration of muscular efforts in attempts to balance than to paresis. In twelve of the animals, including eleven of the above, there were greatly exaggerated movements of limbs, and in three, including two of these twelve and one of the nineteen, definite convulsions. Thus, nineteen monkeys showed incoördination and thirteen exaggerated movements or convulsions, the whole twenty animals being included in these two categories.

The incoördination presented itself as inability to maintain the balance easily, and difficulty in quickly adjusting the limbs and hands to the various movements attempted. The exaggerated muscular movements were especially

noticeable when the animal attempted to jump, and became much more pronounced when it was disturbed and excited. Thus, in jumping on to a perch in the cage, the animal might miss the perch and pass below, or pass above and hit its head against the roof of the cage, or lose its hold and tumble off. In other cases, especially when disturbed, the animal would career wildly round the cage, dash its head against the sides, and apparently be unable to control the propulsive movements which it had initiated. Frequent bruising and injury, especially of the head, occurred under these circumstances. Often these movements were almost convulsive in character.

Definite convulsions occurred in three monkeys, and were merely a supreme exaggeration of the muscular movements already described. They were unaccompanied by a definite loss of consciousness. The animal during the attack would lie partly prostrate, or seized by violent contortions would rotate on the bottom of the cage, or possibly attempt to steady itself by efforts at grasping the bars. Such attacks sometimes lasted several minutes, and left the animal exhausted.

Irregular muscular contractions and twitches, apart from more purposive movements of limbs, were noticed in two animals. A general tremor, or a tremor of limbs or of the head, occurred in two cases, the movements in one resembling those of paralysis agitans. Some spasticity or rigidity of the limbs occurred in three and head retraction in five cases. Five of the animals uttered staccato or barking cries.

Paresis or paralysis was noted in fourteen of the twenty monkeys. In three there was slight paresis of a limb, in five there was marked paresis, and in six there appeared to be paralysis of a limb or other part. Two animals showed squint. In three there was ptosis, marked in two cases. The eyesight may possibly have been affected in one monkey. Three monkeys were drowsy, somnolent, or intensely sleepy.

Terminal prostration was manifested in nearly all the animals, varying from a slight degree, wherein there was inability to rise, with some remaining power of movement in a limb or limbs, to a condition so complete that perhaps an intelligent look in the eyes and slight movements of the tail tip or of a hand or foot were the only recognisable indications of life.

The temperature was frequently subnormal towards the end, sometimes markedly so, and in one case fell to 29° C.

#### V. THE DISEASE AS MANIFESTED IN SHEEP.

1. *Incubation period and general course.* Thirteen successful inoculations confirmed by full histological examination were obtained in sheep. In ten of these the virus came from monkeys, and in the remaining three from previously inoculated sheep. As a converse to the conveyance of the disease from the monkey to sheep, the virus was conveyed from a sheep (A. 65, Sh. 3855) to a monkey (A. 66, Monkey 3860), and from this monkey to a series of these animals. All the inoculations were intracerebral under an anaesthetic, and the virus

had been suspended either in 33 per cent. glycerine or in normal saline solution. Also the inoculations were all made within two days of the death of the previously infected host. After an incubation period of three to twelve days the first symptoms of illness were noticed. The duration of the illness until the animal died, or was *in extremis* and killed, ranged from one to five (or seven) days, except in one case which died thirty days after symptoms were first noticed.

The early symptoms were somewhat vague, as might be expected in an animal showing such low mental development. Hanging of the head and disinclination to feed were taken as suspicious; usually there were quiverings of the lips and ears, and sometimes champing of the jaws, suggestive of cud-chewing; occasionally there was dribbling from the mouth, or a mucous discharge from the nose. Later some retraction of the head usually appeared, whilst the animal, still able to stand and walk, tended to circle to one side.

The final stage was sometimes rapid—in one case lasting an hour—and was frequently characterised by convulsions and great respiratory irregularities. Occasionally convulsive seizures occurred earlier in the disease, and the animal temporarily recovered; indeed, some sheep recovered permanently.

The following notes of a case, where death took place soon after the convulsions first occurred, exemplify the early symptoms:

In the morning it was moving about and eating a little. In the afternoon it was seen in the paddock turning slowly in a circle, with its head down as if trying to reach the grass. After making several revolutions it fell on its side and began nibbling, not grass but a small native plant (*Pimelea*) of unattractive appearance. Shortly afterwards its head became retracted and there were slight convulsive movements, whilst the lips and nostrils were trembling and moving irregularly. It was breathing quickly and there were occasional to and fro movements of the forelimbs, less so of the hind limbs. Later the head became markedly retracted, and the limbs rather rigid and partly convulsed. The segments of the hoof of one of the front feet were sometimes widely separated. The animal seemed to be unconscious. More definite convulsive movements occurred occasionally. At the end of one of these, respirations became highly irregular and then ceased, and the animal died an hour after it had been noticed circling round.

2. *Analysis of the symptoms and signs met with in the sheep.* The following summary deals only with the thirteen sheep in which histological examination proved the presence of "X disease." Those that showed slight symptoms, apparently of "X disease," and recovered, are not included.

Convulsive movements or fits, sometimes intense, occurred in eleven of the thirteen sheep. During these attacks the head was thrown back, the limbs were moved convulsively and sometimes pawed the air, and fine twitchings occurred in the lips, nostrils, and ears. In five cases, before the development of more untoward symptoms, the sheep tended to walk in a circle. Quivering or fine tremors of the lips, ears, nostrils, etc., sometimes extending to the whole

body, occurred in nine cases, and were frequently seen, apart from attendant convulsions. Under similar circumstances, champing movements of the jaws were noticed in two cases, and grinding of the teeth in another. Stiff neck or retraction of the head was noticed during the course of the illness in six cases. In two, the legs were rigid or there was a stiff, jerky gait. Protrusion of the tongue occurred in one animal, "staggers" in one, and restlessness in one. One animal was noted as being drowsy. In most cases terminal unconsciousness or coma accompanied the convulsions. Some weakness of the hind legs was seen in one case. In nine instances rapid or irregular breathing occurred during the course of the illness. Sometimes a series of very rapid respirations were followed by a pause, and then the respirations began again. Other interesting signs were running of the nose or a mucous discharge in two sheep, and dribbling from the mouth in two others.

3. *Sheep surviving intracerebral inoculation, but showing slight symptoms, probably of encephalo-myelitis.* A. 82, Sh. 3894. On June 17th this animal was inoculated intracerebrally with the same material as successfully conveyed the disease on the same date to two other sheep, A. 80 and A. 81. Twelve days later the animal seemed ill and was lying down, and was "off its food," but showed no paresis. Beyond these slight symptoms on this date it remained well until July 6th, when blood was taken from it under an anaesthetic. Four days later it died, apparently from the after-effects of the anaesthetic. No histological lesions of the disease were detected.

It is possible that the slight symptoms shown on one day were due to a very mild attack of encephalitis from which the animal recovered, and that the lesions had disappeared by the time the animal died, eleven days later. Under any circumstances the series of experiments to which this case belongs shows that A. 82 possessed a decided relative, if not an absolute, immunity to the disease.

A. 90, Sh. 3903. On June 27th this animal was inoculated intracerebrally with material that gave a positive result in the case of two other sheep, A. 89 and A. 91. On July 2nd it was breathing fast, held its head down, was drowsy and did not eat. On July 3rd it held its head down when resting, was not eating, and did not seem to run about so much or so quickly as usual, while the hind legs seemed to go down easily when pressure was exerted on the back. On July 4th it was still sluggish and not eating, but next day it was well and remained so afterwards.

This animal had been previously inoculated, under the designation A. 68, with material from a positive sheep, which successfully infected the monkey, A. 66, done on the same date. It is possible that the first inoculation, intended to be intracerebral, was made into the frontal sinus, at any rate it was resultless. The sheep had also received a second inoculation, under the designation of A. 86, from Sh. A. 75, which upon histological examination was found not to show the lesions of encephalo-myelitis.

It seems highly probable that the symptoms from which this sheep suffered after the third injection were due to a mild attack of encephalomyelitis from which it recovered. And it can hardly be denied, especially when its previous inoculations are considered, that the animal possessed a relative, if not an absolute, immunity to the virus.

A. 102, Sh. 3914. On July 7th this animal was inoculated intracerebrally with material which successfully conveyed the disease to two sheep A. 103 and A. 105, and to a monkey, A. 100. It became ill on July 12th and had a temperature of 106° F. On July 13th the temperature was lower, 105°, and from then to the 16th it remained well excepting for occasional twitchings and rapid breathing. It seemed, however, very weak and was "gone in the legs," and the nose was running. On July 19th it seemed well.

Blood was taken under an anaesthetic on July 31st. The serum from this blood was used, on August 2nd, for mixing with a virus before its injection into A. 117, M. 3937. This monkey developed the disease twenty-three days later, which is an unusually long incubation period.

The symptoms presented by this sheep suggest that it may have had a mild attack of encephalitis, from which it recovered. This is perhaps supported by the unduly prolonged incubation period resulting in monkey A. 117, which may be possibly attributed to some immunising power possessed by the sheep serum. The course of the disease in the monkey, however, when it developed, was not altered.

A. 125, Sh. 3948. On August 14th this animal was inoculated intracerebrally with an emulsion from a positive sheep, A. 121. Next day it was a little sick, as the result of the operation. On August 18th it seemed cramped and could not use its hindquarters for a few minutes, and then seemed to be convulsed. On August 19th it showed convulsive movements, lasting for about two hours. Thereafter it remained well until August 25th when one hind leg seemed to be slightly contracted and spastic. It was well next day and remained so afterwards.

This sheep had received a previous inoculation intracerebrally on July 7th with material from a monkey, which produced the disease on the same date in two other sheep, A. 103 and A. 105, and in a monkey, A. 100. It had been unaffected by this first inoculation.

It is possible that the symptoms shown by this sheep, A. 125, were due to a mild attack of encephalitis. The result of the first inoculation, however, shows that it must have possessed a marked relative, if not an absolute, immunity to the virus. If the symptoms manifested after the second inoculation were not due to a mild attack of encephalitis, the result would confirm the view that it possessed absolute immunity. Otherwise a relative or varying immunity would be suggested.

A. 130, Sh. 3968. On August 28th this animal was inoculated intracerebrally with material from a monkey which on the previous day had successfully infected A. 129, M. 3967. On September 5th it seemed sick and was not feeding.

It was walking about, the head was drooping and the respirations were rapid. Next day it seemed worse, was "off its food," and was lying down continually, whilst in the afternoon it was circling round with the head somewhat retracted. On September 7th the lips were twitching at times, and it was shivering all over and had a slight limp in one foreleg. On the afternoon of September 8th it seemed worse, the lips were twitching, it kept poking out its tongue, and was circling round and not eating. On September 9th it had two fits, each of about five minutes' duration, during which it lay down, kicked with its fore and hind legs, and seemed unconscious. On September 10th it was still sick but was able to stand. On being made to move it could walk, but occasionally stumbled and tended to fall on one side. From this date it remained well.

This sheep, under the designation of A. 119, on August 2nd had received a previous inoculation, intracerebrally, of a Berkefeld filtrate of monkey material. It had been unaffected by this first inoculation.

The symptoms manifested by this sheep are strongly suggestive of a mild form of encephalitis, from which it recovered. It would appear that the Berkefeld filtrate contained no virus or a sub-infective dose. It is possible that the injection of this filtrate produced some immunity against the second inoculation, leading to the recovery of the sheep, but this is very doubtful.

A. 131, Sh. 3969. On August 28th this sheep was inoculated in the same way and with the same material as A. 130. It had also received a previous inoculation on August 2nd with the same Berkefeld filtrate. On September 5th the animal showed some paresis of the left foreleg, but could run about and was still feeding. The respirations were rapid. Next day it seemed worse. On September 7th it seemed better and could run about and feed. On September 10th it seemed well except for a little stiffness in one hind leg. At no time did it show any twitchings or head retraction.

It is possible, though doubtful, that the slight symptoms shown by this sheep were due to a mild attack of encephalo-myelitis. The remark under A. 130 again applies.

*Comment.* The histories of A. 125 and A. 130 are distinctly suggestive of mild attacks of encephalo-myelitis. Those of A. 90, A. 102 and A. 131 are also suggestive but less so. In the other sheep, A. 82, the result is very uncertain.

If some of these sheep were "mild takes," then there is evidence of bridging between the fatal disease conveyed to some individual sheep and the complete immunity possessed by others.

4. *Inoculation of sheep in series.—Insusceptibility of some animals.* In four instances, series of sheep, five or six in number, were all inoculated at the same time, in the same way, and under the same conditions, with materials consisting of various parts of the brain and spinal cord of successfully inoculated monkeys. The material used varied according to circumstances, but in most cases was a mixed emulsion of various portions of the brain and spinal cord, and in other cases tissue from the frontal and occipital regions only, or from

the medulla alone. In one series the emulsion was treated with sheep sera. In two animals of another series a Berkefeld filtrate of the emulsion was used. The general results obtained from these four series show conclusively that the material might in one individual sheep produce the typical disease, whilst the same material introduced in exactly the same way, at the same time and under the same conditions in another sheep might fail to produce any illness whatsoever. In several instances reinoculations of these apparently immune sheep with material presumably virulent again failed, suggesting, as is indicated in other experiments, that the immunity thus possessed is a real one, and that the occurrence of non-infection in the series was not due to any over-looked inhibitive factor. Therefore it must be considered as established that some individual sheep are susceptible to the virus and develop the disease when the virus is actually introduced into the middle of the cerebrum, while other individual sheep fail to react in any noticeable way after the virus has been so introduced. These results are very important inasmuch as the inference to be drawn from them may, with some reason, be applied to human beings, and may explain why some persons are attacked with the disease while others, apparently equally exposed to the virus, remain unaffected. Hitherto the general opinion as regards "acute poliomyelitis" may be said to have been that many individuals in the community harboured the virus in their naso-pharynx or in some other situation, and that the virus only occasionally gained access to the central nervous system. In other words, it appeared possible that any carrier might contract this disease, provided immunity had not been established, if the organism should gain entrance to the central nervous system. The results of these sheep inoculations show, however, that even after the virus has been introduced into the central nervous system infection may not necessarily result, but that individuals of the species may be absolutely insusceptible to the virus in spite of not having been already immunised to it.

The following is an account of the results of the four series of sheep inoculations mentioned:

(1) A. 80 to A. 85, Sheep 3892 to 3897. The first four of these animals received an inoculation of material from the usual selected areas of the brain and spinal cord; the fifth, material from the frontal and occipital areas of the brain only; and the sixth from the medulla alone. The last-mentioned animal died within three days of the operation, leaving five animals to be accounted for. The sheep inoculated with the frontal and occipital cortex only remained unaffected and later, under the designation A. 109, Sh. 3921, was successfully inoculated with the disease. The inference to be drawn is that in this particular case the material from the frontal and occipital regions of A. 72, Monkey 3873, either did not contain the virus or contained it in a sub-infective amount. Of the four sheep inoculated with the general emulsion, two developed the typical disease, one was unaffected, and one died 23 days after the operation without having shown definite signs of the disease, and without showing the specific histological lesions.



*Summary.* Of four sheep inoculated with the same material, at the same time and under the same conditions, two developed the disease and two did not.

(2) A. 89 to A. 94, Sheep 3902 to 3907. All these animals were inoculated with material from A. 78, Monkey 3890. The first three received the usual mixed emulsion of various parts of the brain and spinal cord; the fourth and fifth, material from the medulla alone; and the sixth material from the frontal and occipital regions only. Of these animals, two of those receiving the general emulsion and one of those receiving the medulla alone developed the disease, while the other three sheep were unaffected.

*Summary.* Neglecting the sheep which received material from the frontal and occipital regions and for which there was no control to show that this material contained the virus, we find that of five sheep in which the virus was introduced into the brain, three developed the disease and two failed to do so.

(3) A. 101 to A. 106, Sheep 3913 to 3918. These animals received an emulsion of the usual parts of the brain and spinal cord of A. 87, Monkey 3900. This emulsion was mixed with normal saline solution for the injections into two sheep; with the serum of a previously inoculated but unaffected sheep for two others; and with the serum of a normal healthy sheep for the remaining two. One of the last-mentioned sheep died in five days before any symptoms were likely to have manifested themselves. The two receiving the emulsion and saline were unaffected, but one of each of the other pairs developed the disease.

*Summary.* Of five sheep in which the virus was introduced into the brain, two developed the disease and three were unaffected.

(4) A. 119 to A. 123, Sheep 3939 to 3943. These animals were treated with material from A. 110, Monkey 3925. The first two received a Berkefeld filtrate and were unaffected; the other three received an emulsion of the usual parts of the brain and spinal cord. Of these, one developed the disease and two were unaffected.

*Summary.* Of three sheep in which the unfiltered virus was introduced into the brain, one developed the disease and two were unaffected.

5. *Sheep showing natural immunity to intracerebral inoculation of the virus.* Eight sheep, viz. A. 63, (76, 93, 115), A. 69 (94), A. 83 (114), A. 101 (125), A. 104 (126), A. 111, A. 122 and A. 68 (86, 90), are shown, in Appendices XX, XXI and XXV, to have been naturally immune to the disease even when the virus was introduced actually into the brain.

## VI. THE DISEASE AS MANIFESTED IN THE CALF.

The disease was conveyed successfully to one calf, as proved by histological examination after death. In two other cases slight symptoms arose, and in one were almost certainly those of encephalo-myelitis. Both of these animals recovered.

The chief symptoms in the calf, in which the presence of the disease was proved, were restlessness and a tendency to "go" in the front legs, followed by a clear discharge from the nostrils, and on the third day by weakness of the legs and a gait which tended to be circular. The illness terminated in death after general convulsive seizures, with rigidity of limbs, muscular tremors, retraction of the head and arching of the back.

## VII. THE DISEASE AS MANIFESTED IN THE HORSE.

Only one inoculation was made into this species of animal and it was successful, as proved by histological examination. The incubation period was nine days. On the first day of illness the animal tended to move towards one side. The next day there were in addition twitchings of the facial muscles, staring eyes and perhaps partial blindness. On the third day the animal was lying down with the head drawn to one side, and irregular movements of the limbs were observed. This was followed by intense and rapid convulsions, during which the animal was unconscious, alternating with short quiescent periods.

## VIII. OTHER EXPERIMENTS AND VARIOUS INFERENCES DRAWN.

1. *The treatment of the virus with various sera before intracerebral inoculation into monkeys.* Four groups of monkeys pass under this category, viz., A. 9 and 10, A. 20 and 21, A. 27 and 28, and A. 116 and 117.

A. 9 and 10. The serum used in the case of A. 9 was pooled from three children who had had infantile paralysis some time previously. In one case infantile paralysis had occurred  $2\frac{1}{2}$  years before, whilst in the other two cases the disease was probably as remote or more so. The children were under treatment, at the Royal Alexandra Children's Hospital, for the residual paralysis. The serum used in A. 10 was obtained from a healthy medical man who had never suffered from infantile paralysis. The usual emulsion of the human virus was mixed with equal amounts of the respective sera, and kept in an incubator for an hour, and then in the ice-chest over-night. Twenty-four hours after the mixing the injections were made.

A. 8 acts as a control to these two monkeys, inasmuch as the untreated emulsion was inoculated intracerebrally the day previously. A. 8 developed the disease nine days after inoculation, and had a length of illness of six days: A. 9 developed the disease twelve days after inoculation, and had a length of illness of eleven days; and A. 10 developed the disease fifteen days after

inoculation, and had a length of illness of three days. The three monkeys were killed *in extremis*.

From these results it would appear that, by the method employed, the sera of cases which had had infantile paralysis two or three years previously were not capable of neutralising the virus of encephalo-myelitis ("X disease"). It is interesting to note, however, that the two monkeys inoculated with the treated virus had a much longer incubation period than the one inoculated with the untreated virus; and as remarkable that A. 10, in which normal serum was used, had a longer incubation period than A. 9, in which pooled sera, wherein protected bodies might have been expected, were used. It may be noted, however, that whilst the disease in A. 10 ran a rapid course after a long incubation period, in A. 9 it ran a very slow course of eleven days, after a somewhat shorter incubation period. It may be further noted that A. 117, another monkey in which the emulsion was treated with a serum, had a phenomenally long incubation period of twenty-three days; on the other hand A. 124, inoculated with an untreated emulsion, had an incubation period of seventeen days.

*Conclusions.* The treatment of the emulsion of the virus with human serum may possibly lengthen the incubation period. Sera obtained from old cases of infantile paralysis were not proved capable of annulling the virus.

A. 20 and 21. These inoculations need not be further discussed, inasmuch as the emulsion employed was obtained from a monkey which histologically showed no evidence of encephalo-myelitis.

A. 27 and 28. The serum employed in the case of A. 27 was derived from a patient who was believed to have had "X disease" in the previous year. The blood had been forwarded by train and the serum kept in an ice-chest for a few days before use. In the case of A. 28, the serum was a normal human one. The method employed was that already described in connection with A. 9 and 10. Both monkeys failed to "take." As there were no control successful "takes" with material from this human case, there is unfortunately no evidence that the virus was present in the emulsion. Therefore nothing can be learned from the experiments.

A. 116 and 117. These monkeys received injections of a virus from A. 110, Monkey 3925. The virus consisted of an emulsion in normal saline solution of tissues from the usual regions in the brain and spinal cord. After being emulsified, the material was centrifuged at a low speed for two minutes, which yielded a supernatant milky fluid and a deposit of coarser fragments. The fluid was divided into two portions, and to each an equal amount of one of the sera to be mentioned was added. The mixtures were shaken, then incubated for two hours, then kept at room temperature for an hour, and finally injected intracerebrally into the respective monkeys. The object of this procedure was to see whether, during the time given and with the amount of serum employed, the infectivity of the virus would be annulled.

A. 116, Monkey 3936, received the supposed virus mixed with serum from

A. 89, Sheep 3902. This sheep was inoculated on June 27th with material from a previous monkey. It showed slight symptoms on July 2nd, 3rd and 4th, which might be considered as indicating a mild form of encephalo-myelitis. On July 5th it seemed well again. On July 18th 20 c.c. of blood were removed under an anaesthetic. It was used for immunity experiments on sheep. The animal was bled again on July 31st under an anaesthetic. Two days later it was sick, apparently from lung trouble, was worse next day and died on August 4th. Histological examination showed lesions of encephalo-myelitis, apparently in a stage of early resolution, suggesting that the symptoms manifested from July 2nd to 4th were due to a mild form of this disease from which the animal was in process of recovery. A. 116, Monkey 3936, remained unaffected by the inoculation of the virus combined with the serum of this sheep.

A. 117, Monkey 3937, received the virus intermixed with the serum of A. 102, Sheep 3914. This sheep had been inoculated on July 7th with material from a previous sheep, A. 101, Sh. 3913. It became ill five days later with a raised temperature, and thereafter had occasional twitchings, breathed rapidly, seemed very weak, was "gone" in the legs, and had a running nose. It appeared perfectly well on July 19th. Blood was withdrawn under an anaesthetic on July 31st. The symptoms manifested by this sheep suggest that it may have had a mild form of encephalo-myelitis from which recovery had resulted. A. 117, Monkey 3937, was found prostrate on the bottom of the cage twenty-three days after the inoculation. It exhibited intense incoördinated movements on being disturbed, and histological examination after death showed typical lesions of encephalo-myelitis.

*Discussion of the results.* First it is necessary to point out that the monkey supplying the virus employed in these two experiments, namely A. 110, Monkey 3925, at the time it was killed appeared as though it might have recovered from the disease. The possibility, therefore, is that the strain of virus at this stage possessed less virulence than it had originally, so that even without any other treatment it might have failed to convey the disease to a monkey, or have only produced the disease after a long incubation period, or in mild form, when it did appear. The results show that A. 116 did not contract the disease and that A. 117 contracted it after an unduly prolonged incubation period of twenty-three days. It is to be noted, however, that when the disease did arise in A. 117, it appeared in an intense form. After making due allowance, therefore, for the possibility that the activity of the virus was waning, the negative result in A. 116 is in support of the view that the serum of A. 89, Sheep 3902, actually did neutralise the virus under the circumstances of the experiment. It might have been expected, moreover, that the serum from this sheep, which was actually shown later to have suffered from encephalo-myelitis, would have possessed immune bodies—if such are developed in this disease—at the time when it was withdrawn. As regards A. 117, the inferences are less clear. The sheep whose serum was employed may or may not have had encephalo-myelitis. The unduly prolonged incubation period may or may not have

been the expression of some immune properties held by this serum. The serum may in fact have delayed the appearance of the disease without modifying its course when it did appear.

2. *The treatment of the virus with various sera before intracerebral inoculation into sheep.* Four sheep, A. 103, 104, 105 and 106, were inoculated with material from A. 87, Monkey 3900. The usual emulsion in normal saline solution was centrifuged at a low speed for about seven minutes. The supernatant fluid was then divided into two portions and each mixed with an equal amount of serum— with serum from a normal sheep, in the case of the last two, and with serum from A. 82 Sheep 3984, in the case of the first two animals. After incubation for two hours, the mixtures were kept at room temperature for a further two hours, and then injected intracerebrally into the respective sheep.

The serum-yielding sheep, A. 82, had been inoculated with material from A. 72, Monkey 3873, on June 17th. On June 29th it seemed ill but showed no paresis. It was bled under an anaesthetic on July 6th for the purpose of the present experiments and died on July 10th, apparently from the after-effects of the anaesthetic. Histological examination showed no lesions of encephalo-myelitis. It should be noted that two companion sheep to A. 82, viz., A. 80 and A. 81, both developed the disease after identical intracerebral inoculations.

A. 103, Sheep 3915, developed the disease, whilst A. 104, Sheep 3916, showed no symptoms, being probably an immune animal.

A. 105, in which the emulsion was treated with serum from a normal sheep, developed the disease, but A. 106, similarly treated, died from lung trouble in five to six days, perhaps before signs of the disease could have manifested themselves.

*Discussion of results.* The serum of A. 82, Sh. 3894, failed to neutralise the virus in the method employed when injected into A. 103, Sh. 3915. As explaining this result, there is no evidence that A. 82, Sh. 3894, actually had encephalo-myelitis, so as to be in the position of possessing anti-bodies in its serum.

The positive results in A. 105, together with the positive results in A. 103, both show that by the method employed sheep serum alone does not neutralise the virus.

A. 111, 112 and 113. As regards these inoculations, the procedure was that adopted in the preceding series, save that the mixtures were incubated for only 1½ hours and were inoculated an hour later. Again, as showing that the virus was present in the material used, A. 110, Monkey 3925, acts as a control since this received an injection differing only in the replacing of the serum by an equal amount of normal saline solution, and the animal developed the disease. The serum employed for the three sheep was that from A. 89, Sheep 3902, taken on July 18th. A short summary of the history of A. 89 has been already given in discussing the experiments on the two monkeys, A. 116 and

117, in the preceding section. It will be remembered that the serum employed in the case of these monkeys, and which apparently protected A. 116 against infection, was obtained from A. 89, Sheep 3902, on July 31st, whereas the serum employed in the case of these sheep was that obtained on July 18th. A. 111 showed no evidence of encephalo-myelitis after its inoculation; the other two animals died two and four days respectively after the inoculation before any symptoms could possibly have arisen.

*Discussion.* It is possible that A. 111, Sheep 3923, was protected against infection by the blood serum of A. 89, Sheep 3902. As, however, many sheep are naturally immune to the intracerebral injection of the virus, the question of the protective value of this serum at this date is by no means proved.

3. *Experiments suggesting that the virus is no longer present on the 8th or 10th day of illness in monkeys.* In the discussion on the "Failure in Monkeys of Certain Intracerebral Inoculations of Brain and Spinal Cord from Monkeys" (vide Appendix VI) it is suggested that the failure to infect A. 18, Monkey 3823, and perhaps A. 19, Monkey 3824, was due to the disappearance of the virus during the eight or ten days of illness that had elapsed before the death of A. 9, Monkey 3785.

4. *Experiments showing presumed dying out of the virus during prolonged storage in glycerine in the cold.* A. 17, Monkey 3836, was inoculated with material from a monkey on April 11th and was unaffected thereby. The same material on February 13th had successfully conveyed the disease to A. 14, Monkey 3805. That A. 17 was not immune was shown by its successful inoculation later as A. 55, Monkey 3848. The inference is that the virus had died out during the storage for two months in glycerine emulsion in the cold.

A. 96, Sheep 3935 was inoculated on July 29th with material from a monkey, A. 78, Monkey 3890, and remained unaffected. The same material on June 27th and 28th had successfully conveyed the disease to a monkey, a sheep and a horse. This sheep, A. 96, was not further tested to see whether it possessed natural immunity. The failure of the experiment may therefore be attributed either to the length of time the material was preserved in glycerine in the ice-chest, namely a month, or to natural immunity.

5. *Experiments possibly showing a waning in virulence of the virus.* A. 116, Monkey 3936, was inoculated with monkey material which had been treated with the serum of a "positive" sheep, A. 89. The monkey was unaffected by the inoculation, whilst another monkey, A. 117, M. 3937, which was inoculated on the same day with the same material, save the substitution of serum from another sheep, A. 102, developed the disease after an unduly prolonged incubation period of 23 days, the disease thereafter running its usual course. It is probable that the failure of A. 116 to "take" was attributable to the neutralising power of the sheep serum, and it is possible that the prolonged incubation period in A. 117 was due to the presence of similar neutralising bodies, but

to a less degree, in the second sheep's serum. On the other hand it is possible that the negative result in the first monkey, and the prolonged incubation period in the second, were due to a waning in activity of the virus leading to complete failure to "take" in some monkeys and a prolonged incubation period in others. It may be noted that A. 132, which is the same monkey as A. 116, also escaped disease when inoculated later with material from another positive monkey. This second failure may be attributed either to the animal having been rendered artificially immune by the first inoculation, or to the supposed waning of virulence leading to a "take" in some monkeys and a failure in others.

6. *Experiment suggesting the production of artificial immunity.* A. 132, Monkey 3977, which has just been discussed, may be an instance of artificial immunity resulting from the administration of an active virus which had been exposed to the serum of a sheep, which sheep had had encephalo-myelitis and still showed lesions of this disease.

#### IX. SUMMARY AND CONCLUSIONS.

1. The disease is an acute encephalo-myelitis produced by a virus akin to, but not identical with, that of the Heine-Medin disease.

2. The disease was readily communicated, with fatal results, to monkeys (*Macacus rhesus*) by intracerebral inoculation of a suitably-prepared emulsion of nervous substance (brain, cerebellum, pons, medulla and spinal cord) from the human subject dead from "X disease." Moreover, the virus was found to breed true in a succession of thirteen monkey (*Macacus rhesus*) generations.

3. The disease was not communicated to *Macacus cynomolgus* (several trials).

4. The disease was communicated by the above-mentioned method from monkey to sheep (10 times), from sheep back to monkey and on again from monkey to monkey.

5. A certain number of sheep, perhaps 50 per cent., were found wholly insusceptible to the disease; others suffered lightly and recovered.

6. The disease was communicated, with fatal results, by the same method, from monkey to horse (1 case) and to calf (1 case). Two calves suffered lightly after intracerebral inoculation of the usual virus-containing material taken from monkey and horse respectively.

7. The virus appears to be held back completely, or to a great degree, by the pores of a Berkefeld filter.

8. Storage of the virus-containing material in diluted glycerine, under cool conditions, for longer than a few days, reduced or annulled its nocive properties.

9. Drying of the virus-containing material in Petri dishes, in an incubator, probably destroys its activity.

10. In the case of the sheep, there was failure to induce the disease by swabbing the nostrils with virus-containing emulsion.

11. There is some evidence that in the case of the sheep and the calf a previous inoculation with the virus confers immunity.

12. One experiment suggested that artificial immunity might be induced in the monkey by inoculation of virus treated with serum from an "X disease" sheep.

13. Intracerebral inoculation of three dogs, one kitten, two rabbits and one hen failed to produce any signs of the disease; and similar inoculations of two guinea-pigs gave doubtful results.

14. Treatment of the virus-containing emulsion with (a) normal human serum, (b) serum from recovered human cases of "acute poliomyelitis" and (c) serum from "X disease" sheep prolonged the incubation period of the disease in the monkey but did not destroy the virus.

15. Normal sheep serum and serum from "X disease" sheep did not neutralise the virus in its operation on other sheep.

16. Two experiments suggested that the virus was no longer present in the monkey on the eighth or tenth day of illness.

17. Two experiments towards the end of the investigation suggested a waning in strength of the virus.

18. Intraperitoneal and intrasciatic inoculations of virus-containing material, also intracerebral inoculations of cerebro-spinal fluid, of a filtrate of faeces, of a "Noguchi culture," of an emulsion of fowl ticks, of naso-pharyngeal swabs from human cases and contacts, and inoculations into veins, all failed.

## X. APPENDICES<sup>1</sup>.

### APPENDIX I. *Tabulation of the kinds of Inoculations and Results in the respective Species of Animals.*

Each number refers to an individual experiment on an animal. Since in many instances it was necessary, when no result followed, to use the animal again for a further experiment, it is obvious that the number of animals actually used is considerably less than might be inferred from the number of experiments made.

**MONKEYS:** *Macacus rhesus* and *Macacus cynomolgus* = 62.

Positive results from the intracerebral inoculation of material from three human cases: A. 8, 9, 10, 33, 48 = 5

Positive results from the intracerebral inoculation of material from monkeys: A. 14, 49, 50, 55, 62, 64, 66, 72, 78, 87, 100, 110, 117, 124, 129 = 15

Animals dying shortly after the operation as a direct result of this or from early sepsis. (These cases will not be further considered): A. 7, 13, 22, 25, 47, 51, 128 = 7

<sup>1</sup> Throughout the Appendices the abbreviations M. and Sh. stand for Monkey and Sheep respectively.



Death in 12 days from pathogenic infection, without co-existent evidence of encephalo-myelitis: A. 11	= 1
Failure of intracerebral inoculations of the brain (and spinal cord) from eight human cases: A. 27, 28, 29, 37, 38, 39, 40, 41, 44, 45	= 10
Failure of certain intracerebral inoculations of the brain and spinal cord of monkeys: A. 17, 18, 19, 20, 21, 116, 132	= 7
Failure of intraperitoneal inoculations of human spinal cord: A. 6, 24	= 2
Failure of the intrasciatic inoculation of the brain and spinal cord from a human case: A. 5	= 1
Failure of the intracerebral inoculation of Pasteur-Chamberland F. filtrates: A. 12, 26, 54	= 3
Failure of the intracerebral inoculation of cerebro-spinal fluid: A. 1, 32	= 2
Failure of the intracerebral inoculation of a Pasteur-Chamberland F. filtrate of faeces: A. 31	= 1
Failure of the intracerebral inoculation of a "Noguchi culture"	= 1
Failure of the intracerebral inoculations of naso-pharyngeal swabs from contacts and a case: A. 3, 30	= 2
Failure of the intracerebral inoculation of the brain and spinal cord of a horse: A. 42	= 1
Failure of the intracerebral inoculation of an emulsion of fowl ticks: A. 43	= 1
Failure of the intraperitoneal inoculation of swabs from contacts: A. 2	= 1
Failure of the intrasciatic inoculation of swabs from contacts: A. 4	= 1
Failure of the introduction of the virus into a vein after lumbar puncture: A. 71	= 1

**SHEEP: = 52.**

Positive results from intracerebral inoculation of material from the brain and spinal cord of monkeys: A. 52, 65, 80, 81, 89, 91, 92, 103, 105, 121	= 10
Positive results from intracerebral inoculation of material from the brain and spinal cord of positive sheep: A. 98, 108, 109	= 3
Dying within four and a half days of the operation as a direct result of this, or from post-anaesthetic lung trouble or sepsis: A. 85, 99, 112, 113, 123	= 5
Surviving intracerebral inoculation but showing slight symptoms, possibly of encephalo-myelitis: A. 82, 90, 102, 125, 130, 131	= 6
Showing no symptoms after intracerebral inoculation of brain and spinal cord from human cases: A. 35, 46	= 2
Showing no symptoms after intracerebral inoculation of brain and spinal cord from infected monkeys: A. 53, 63, 69, 75, 83, 84, 93, 94, 96, 101, 104, 106, 111, 122	= 14
Showing no symptoms after intracerebral inoculation of brain and spinal cord from infected sheep: A. 68, 114, 115, 126	= 4
Showing no symptoms after intracerebral inoculation from a monkey histologically negative. (This case will not be further discussed): A. 23	= 1
Showing no symptoms after intracerebral inoculation from a sheep histologically negative. (This case will not be further discussed): A. 86	= 1
Showing no symptoms after swabbing the nose with virus-containing material: A. 70	= 1

- Showing no symptoms after the introduction of horse serum into the spinal canal and of a small quantity of virus into a vein: A. 76 = 1
- Showing no symptoms after intraperitoneal inoculation of the virus: A. 67 = 1
- Showing no symptoms after the intracerebral injection of dried tissue from the brain and spinal cord: A. 77 = 1
- Showing no symptoms after intracerebral inoculation of a Berkefeld filtrate of virus-containing material: A. 119, 120 = 2

**CALVES: = 4.**

- Positive result after intracerebral inoculation with material from the brain and spinal cord of a monkey: A. 57 = 1
- Showing symptoms of illness, possibly of encephalo-myelitis, after intracerebral inoculation of material from the brain and spinal cord of a positive monkey or a positive horse: A. 88, 107 = 2
- Showing no symptoms after intracerebral inoculation (a second inoculation) with brain and spinal cord from a positive sheep: A. 127 = 1

**HORSE: = 1.**

- Positive result from intracerebral inoculation of material from the brain and spinal cord of a monkey: A. 95 = 1

**DOGS: = 5.**

- Showing no symptoms after an intracerebral inoculation of material from the brain and spinal cord of a human case, a monkey case, or a sheep case: A. 16, 34, 56, 97 = 4
- Dying as a result of the operation. (This case will not be further discussed): A. 15 = 1

**KITTENS: = 2.**

- Showing no symptoms after intracerebral inoculation of brain and spinal cord from a positive monkey: A. 79 = 1
- Dying as the result of the operation. (This case will not be further discussed): A. 61 = 1

**RABBITS: = 3.**

- Showing no symptoms after intracerebral inoculations: A. 58, 73 = 2
- Dying as the result of the operation. (This case will not be further discussed): A. 59 = 1

**GUINEA-PIGS: = 2.**

- Showing indefinite symptoms and doubtful histological results after intracerebral inoculation of brain and spinal cord from infected monkeys: A. 60, 74 = 2

**HEN: = 1.**

- Showing no symptoms after intracerebral inoculation of brain and spinal cord from an infected monkey: A. 118 = 1.

APPENDIX II. Table of Monkey Inoculations. Positive Results.

No of Monkey	Date	Source of material	Day of illness when virus was obtained	Hours after death when p.m. examination made	Period virus was in vitro	Menstruum in which virus was preserved	Incubation period in days	Length of illness in days
A. 8, M. 3783	29/1/18	Case 27, Narrabri	3	21	1 day	33% glyc. in saline	9	6
A. 9, M. 3785	30/1/18	" 27 "	3		2 days		12	11
A. 10, M. 3786	30/1/18	" 27 "	3		2 days		15	3
A. 14, M. 3805	13/2/18	A. 8, M. 3783	6	Immediate	1 day	50 % glyc. in saline	10	3
A. 33, M. 3803	13/2/18	Case 38, Wee Waa	2	12	1 day	33 % "	12	6
A. 48, M. 3829	22/3/18	Case 32, Narrabri	5	5 hours	7 days	33 % glyc. sol. in saline	11	3
A. 49, M. 3835	4/4/18	A. 48, M. 3829	3	Immediate	A few hours	Normal saline	7	5
A. 50, M. 3839	15/4/18	A. 49, M. 3835	5	"	"	33 % glyc. sol.	5	5
A. 55, M. 3848	16/4/18	A. 50, M. 3839	5	Some hours	1 day	"	8	2½
A. 62, M. 3845	23/4/18	A. 52, Sh. 3839 b	4	3	1 day	"	5	6
A. 64, M. 3854	7/5/18	A. 55, M. 3848	2½	A few hours	2 days	"	7	5
A. 66, M. 3860	17/5/18	A. 65, Sh. 3855	1	A few hours	1 day	"	6	8
A. 72, M. 3873	30/5/18	A. 66, M. 3860	8	Immediate	A few hours	Normal saline	11	6
A. 78, M. 3890	15/6/18	A. 72, M. 3873	6	"	"	"	10	3
A. 87, M. 3900	27/6/18	A. 78, M. 3890	3	"	"	"	7	4
A. 100, M. 3912	7/7/18	A. 87, M. 3900	4	"	3 hours	"	9	4
A. 110, M. 3925	19/7/18	A. 100, M. 3912	4	"	A few hours	"	11	4*
A. 117, M. 3937	2/8/18	A. 110, M. 3925	4	"	"	"	23	3
A. 124, M. 3952	17/8/18	A. 110, M. 3925	4	"	15 days	33 % glyc.	17	5
A. 129, M. 3967	27/8/18	A. 117, M. 3937	3	"	A few hours	Normal saline	10	4

\* Perhaps would have recovered.

M. = Monkey. Sh. = Sheep.

APPENDIX III. *Details of the Successful Inoculations in Individual Monkeys (Macacus rhesus).*

A. 8, M. 3783, became ill on the ninth day. During the first two days its movements were violent and incoördinate, and it showed apparent weakness in the legs. On the third day it was quieter, but markedly weak and very clumsy in its movements. On the sixth day, when it was killed, both legs were markedly paretic, whilst the right arm seemed absolutely paralysed and useless.

A. 9, M. 3785, became ill on the twelfth day, showing slight weakness in the legs. Its condition was much the same on the two following days. On the fourth day the right arm seemed decidedly paretic and clumsy, whilst the legs were markedly paretic and dragged after the body on movement. The body swayed on moving and movements were clumsy. On the fifth day the animal was ataxic and frequently fell over, whilst its movements were clumsy and the neck seemed weak. Its condition during the next three days was much the same. On the ninth day, in addition, the head was somewhat retracted; the muscles of both arms and legs still possessed some tone. On the eleventh day, when the animal was killed, it was much exhausted and no movements could be detected in the arms.

A. 10, M. 3786, after an incubation period of fifteen days, showed an anxious expression and seemed drowsy. It reeled like a drunken man; the arms and legs seemed paretic. On the second day of illness it showed marked ataxia and the neck seemed weak. On the third day it was lying on its side; the right arm was apparently paralysed; the left arm and both legs could be moved a little, but some tone still remained in the limbs. The animal was killed on this day.

A. 14, M. 3805, after an incubation period of ten days, became markedly ataxic whilst the hind limbs were apparently paretic. Next day it uttered sharp cries from time to time, and its head was frequently buried on the chest. On being startled it reeled and swayed about in a convulsive way, injuring itself. The hind limbs and right arm were weak. On the third day the animal was quite prostrate; it showed slight convulsive movements of the left arm and left leg, and the head was slightly retracted. The temperature was subnormal. It died on this day.

A. 33, M. 3803, after an incubation period of twelve days, had an anxious expression and showed clumsy movements. There was no definite paresis. On being disturbed, convulsive seizures, without loss of consciousness, occurred. On the second day there was marked incoördination and apparently some rigidity, whilst attacks of convulsive movements occurred from time to time. There was marked incoördination and an occasional twitch of the limbs. Partial paresis of the hands was manifested when it tried to feed itself. The same intense incoördination and general convulsive movements occurred during the next two days. On the fifth day it was lying on its side at the bottom of the cage; both hind limbs seemed useless but rather rigid than flaccid, whilst both arms were paretic. On the sixth day, when it was killed, the animal was still prostrate, and there was paresis of all four limbs which were rather flaccid.

A. 48, M. 3829, became ill on the eleventh day and showed the usual very anxious expression. Its movements were decidedly incoördinate. During the next two days the incoördination was greatly increased and an internal squint was present on the third day of illness when the animal was killed.

A. 49, M. 3835, became ill on the seventh day, showing a frightened expression and being nervous. One arm and leg seemed rather spastic. On the third day of illness it uttered peculiar staccato cries, was very "jumpy," and showed exaggerated incoördinated movements. Next day its condition was worse, and on the fifth day, when it was killed, it was prostrate on the bottom of the cage with the head markedly retracted.

A. 50, M. 3839, showed a frightened expression on the fifth and sixth days after its inoculation. On the third day of illness it uttered barking noises; its movements were exaggerated but there was no definite incoördination. Next day, in addition, there was slight incoördination, and on the fifth day of illness the animal was prostrate on the bottom of the cage, though it could partly raise itself. Its movements were much exaggerated, during which it injured itself. It was killed with chloroform on this day.

A. 55, M. 3848, did not use the left arm on the seventh day after its inoculation. Next day its movements were very shaky and rather incoördinate. This may be considered as the first day of illness. On the second day the animal was intensely shaky, trembling all over as if from paralysis agitans, and was rather incoördinate in its movements; it died during the night.

A. 62, M. 3845, on the fifth day after inoculation appeared to show slight incoördination. Next day its movements were distinctly ataxic, and there appeared to be some weakness in one arm and leg. On the third day it swayed on movement, but the apparent weakness of the arm and leg had disappeared. On the fourth day it was much the same. On the fifth day the hind limbs seemed paretic and movements were very shaky, jumpy, and incoördinate. The animal fell from time to time on the bottom of the cage, and showed violent incoördinated movements, almost convulsive in character. The animal was killed with chloroform.

A. 64, M. 3851, after an incubation period of seven days, seemed jumpy when disturbed. Two days later it looked anxious, and there was marked incoördination of movement. On the fourth day it was lying prostrate on the bottom of the cage. It moved the arms a little but the movements were incoördinate and paretic. Next day the animal could not sit up or even hold its head up, and the head was slightly retracted. There were slight irregular muscular contractions in the right arm, both legs and the tail. Some tone was still retained in the limbs. Chloroform was administered.

A. 66, M. 3860, after an incubation period of six days, had an anxious expression and its movements were excessive and there was slight incoördination. Next day, in addition, there was weakness of the hind limbs. From the third to the fifth day it was much the same, but on the sixth day the right arm was apparently paretic. On the seventh day it was worse, but could still jump about and feed. On the eighth day it was prostrate on the floor of the cage, and there seemed to be almost complete paralysis. On the eighth day the condition was worse, though the limbs were not absolutely flaccid, and still possessed some tone. The animal was killed by chloroform.

A. 72, M. 3873, after an incubation period of eleven days, had an anxious expression. For the next two days its condition was the same, but on the fourth day there was ptosis of both eyelids, slight incoördination of movement, and dragging of the left leg. On the fifth day its movements were markedly incoördinate and jumpy, and there were slight twitchings of the left arm. The right arm and leg during the day became paretic. On the sixth day it was prostrate on the bottom of the cage, and the right side seemed paralysed. This was found not to be a true complete paralysis, as there was some resistance to passive movement. The knee-jerks and elbow-jerks were present. The animal was killed with chloroform.

A. 78, M. 3890, after an incubation period of ten days, had a slightly anxious expression and seemed more excitable than its healthy fellow. Next day it was markedly ill and apparently intensely sleepy. There was definite ptosis on both sides, but more decided on the left side. It swayed on movement and struggled violently. On the third day it was prostrate, could not raise its head or sit up. There was no head retraction; the limbs still possessed tone. Chloroform was administered.

A. 87, M. 3900, after an incubation period of seven days, had an anxious expression and was rather jumpy. Next day its movements were very incoördinate and it had two convulsions after being disturbed. On the third day its condition was worse, and towards evening it was prostrate on the bottom of the cage, and when disturbed, violent and irregular con-

vulsive movements occurred. On the fourth day it was still prostrate; it could only move the arms and tail a little, but some tone was still present in them. Chloroform was administered.

A. 100, M. 3912, after an incubation period of nine days, had a slightly anxious expression. Next day its countenance was still slightly anxious-looking, and there was decided incoördination and exaggeration of movements. On the third day it had fallen to the bottom of the cage, and could only raise itself partly and then struggled round with convulsive movements. The head became retracted. On the fourth day the animal was still prostrate, uttered occasional barking noises and had head retraction. The limbs were rather rigid and there was no flaccid paralysis though there was probably some paresis. Chloroform was administered.

A. 110, M. 3925, after an incubation period of eleven days, had an anxious expression and walked somewhat "gingerly." Next day there was marked incoördination, and it presented a somnolent appearance at times. There was slight tremor of the arms and occasional tremors of the head, limbs and hands. Next day the head was somewhat depressed on the chest and would gradually sink lower and lower as if the animal were dropping off to sleep, when it would overbalance and struggle incoördinately round. There were slight muscular twitchings, and the eyes were occasionally turned to the left; there was drooping of the eyelids. On the fourth day the monkey seemed distinctly, though slightly, better. There was an inward and upward squint of the right eye, but no ptosis of either lid. It was not now somnolent. Its movements were distinctly incoördinate and violent. There was no definite paresis. The animal looked as though it might possibly recover. Chloroform was administered to obtain the virus.

A. 117, M. 3937, after an incubation period of twenty-three days, was found prostrate on the bottom of its cage. On being disturbed it struggled round with intense incoördinate movements, during which it injured itself. It uttered short sharp barking noises from time to time. It could not sit up or stand, but could grip hold with its hands and feet. On the second day of illness it was quite prostrate but conscious; the right arm seemed paralysed, but it kicked vigorously with both hind legs and the left arm. On the third day of illness, when chloroform was administered, the right arm was severely paralysed though there was still some slight tone in the biceps and very slight tone in the flexors of the forearm. The left arm was markedly paretic and rather rigid, whilst the legs were rather rigid and paretic. The knee and back muscles seemed also paretic. The temperature was subnormal.

A. 124, M. 3952, after an incubation period of seventeen days, was noticed to be slightly "nervous." Next day the left arm and leg were weak, and the animal was still "nervous." On the third day the left arm seemed quite paralysed whilst the left leg and the hind-quarters were weak. It did not seem able to see things properly. On the fourth day it was having convulsions at frequent intervals, lying on the floor of the cage between times. On the fifth day, that in which the animal was killed, the left arm seemed completely paralysed, but it could grip with the right hand and both feet. The temperature was subnormal.

A. 129, M. 3967, after an incubation period of ten days, appeared "nervous" with incoördination of movement and some paresis of the left arm. Next day there was intense incoördination, during which the animal injured itself against the sides of the cage; some of its muscular movements might be described as contortions. On the third day of illness the left arm appeared to be completely paralysed, and there was indefinite weakness of the right arm. Convulsions, lasting a few seconds, occurred from time to time. Incoördination was intense. On the fourth day the animal was found prostrate on the bottom of its cage apparently dead. Its eyes still seemed to recognise its surroundings, however, whilst there were slight indications of movement at the ends of the extremities. The temperature was markedly subnormal.

#### APPENDIX IV. *Death of a Monkey in Twelve Days from Pathogenic Infection without Co-existent Evidence of Encephalo-myelitis.*

A. 11, M. 3801, was inoculated on February 12th, with material from A. 8, M. 3783, which was killed on this date. A day later A. 14, M. 3805, received an inoculation containing the same virus with the addition of serum from the monkey contributing the virus. A. 14 developed encephalo-myelitis, as proved by final histological examination. A. 11, nine days after inoculation, developed symptoms resembling those of other animals infected with this disease. It died four days later. Histological examination showed a picture obscured by widespread miliary abscesses in which micrococci were detected. There was no histological evidence of encephalo-myelitis. As portions of the brain, from increased intracranial pressure, protruded through the trephined hole, and the surface wound, in consequence, had re-opened after the animal became ill, complicating septic infection was expected. It was, however, believed at the time that the hernial protrusion had been caused by the congestion of the brain due to the development of encephalo-myelitis, and that lesions of this disease would be detected as well as those of the secondary infection; such, however, was not the case. The interesting speculation arose as to whether histological lesions were really present but were overlooked or masked, or whether the pyogenic infection had in some way destroyed the virus of encephalo-myelitis. As against the first of these views, it may be noted that the inoculations made from A. 11 were negative; somewhat in favour of the latter, and as tending to show that active virus was originally injected into A. 11, is the successful result with the same material, used on the following day, in A. 14.

#### APPENDIX V. *Failure in Monkeys of Certain Intracerebral Inoculations of Human Material.*

Intracerebral injections of emulsions from portions of the brain and (or) spinal cord from human cases failed as follows:

A. 27, M. (*Mucacus rhesus*) 3809 (from Case 28, J. M., Narrabri, dying on the sixth day), inoculated with the glycerine emulsion of the upper cervical cord treated with serum of Case 30, O'M, 1917 series, a supposed recovered case. Inoculation made ten days after patient's death.

A. 28, M. (*M. rhesus*) 3810, inoculated as in A. 27. M. 3809, but the glycerine emulsion was treated with a normal serum. Inoculation ten days after patient's death.

A. 29, M. (*M. rhesus*) 3819, inoculated with the same material as A. 27, M. 3809, and A. 28, M. 3810, but without treatment with serum, and twenty-eight days after the death of the patient.

A. 37, M. (*M. rhesus*) 3811 (from Case 6, R. M., Broken Hill, dying on the fifth day of the disease), inoculated with a glycerine emulsion of portions of the brain and spinal cord after transmission through the post. Inoculation eight days after patient's death.

A. 38, M. (*M. rhesus*) 3812 (from Case 7, G. B., Broken Hill, dying on the fourth day of the disease), inoculated with a glycerine emulsion of portions of the brain and spinal cord after transmission through the post. Inoculation eight days after patient's death.

A. 39, M. (*M. rhesus*) 3830 (from Case 18, E. C., Broken Hill, dying on the eighth day of the disease), inoculated with similar material to A. 37, M. 3811, and A. 38, M. 3812. Inoculation eleven days after patient's death.

A. 40, M. (*M. rhesus*) 3828 (from a supposed case in Brisbane). The glycerine emulsion of the brain and spinal cord was inoculated a week or ten days after the patient's death.

A. 41, M. (*M. rhesus*) 3831 (from Case 54, G. S., Narrandera, dying on the 12th day of the disease). Small portions of the brain and spinal cord were transmitted through the post in glycerine, and inoculations made five weeks after the patient's death.

A. 44, M. (*Macacus cynomolgus*) 3825 (from Case 37, A. C. F., Boggabri, dying on the third day of the disease, post-mortem next day). A glycerine emulsion of portions of the brain and spinal cord was inoculated two days after the patient's death.

A. 45, M. (*M. rhesus*) 3837, was inoculated with the same material as A. 44, M. 3825, twenty-nine days after the patient's death.

### *Analysis of the above unsuccessful results.*

CASE 28. As regards the experiments contingent on Case 28, J. M., Narrabri, of the two monkeys used for the inoculations, one monkey (A. 28, M. 3810) was two months later successfully inoculated with the disease from A. 50, M. 3839, under the designation of A. 55, M. 3848, on 16/4/18. It would therefore appear that this monkey was susceptible to the disease and that it had not been protected against the later introduction of the virus by having suffered from a mild but unrecognised attack after the first inoculation. Only a very partial post-mortem examination was allowed on this human case, the material obtained being curetted from the upper cervical cord through a small incision in the back of the neck. Failure in the inoculation may be attributed to this particular area of the spinal cord either not containing the virus (though showing the characteristic lesions), or containing the virus in a subinfective amount. The treatment of the emulsions by sera, one of which was normal, cannot be considered as the cause of failure, inasmuch as A. 9, M. 3785, and A. 10, M. 3786, were inoculated with emulsions also treated with sera, and these monkeys "took." It is of course possible that the serum of the supposed recovered case of the previous year might have protected A. 27, M. 3809, had the virus been present. The normal serum, however, should not have protected A. 28, M. 3810. The inoculations were made ten days after the patient's death, and this period of glycerinisation may have been responsible for the failures. As regards A. 29, M. 3819, inoculated with the glycerinated material untreated by serum, twenty-eight days after the patient's death, failure may have again occurred either because of the absence of the virus originally in the material used or on account of the long period during which the virus had been exposed to the influence of glycerine.

*Conclusions.* Failure of inoculations from Case 28, J. M., Narrabri, may be attributed either to the absence, relative or complete, of the virus in the material obtained, or to the length of time, ten to twenty-eight days, during which the glycerinated material was kept.

*Broken Hill and Narrandera Cases.* As regards the four cases in which the material was transmitted by post, we did not take this material ourselves, and it is possible that portions of cerebral tissue containing the virus were not selected. The material came in blocks in glycerine during the warm period of the year, and exposure to the late summer high temperature may have destroyed the virus. The inoculations were made in two instances eight days after death of the host, in one eleven days, and in one five weeks. As the patients died on the 4th, 5th, 8th and 12th days of the disease the virus should have been still present in the brain or cord of two of these cases at least, when death occurred.

*Conclusions.* Failure in these four cases may be attributed to the exposure of the glycerinated material during transit for several days to the high temperature of late summer—a condition inimical to the keeping qualities of vaccinia virus for instance—or to the period of exposure to glycerine after death of the patient, which was eight days or longer; or possibly to infective material not having been selected.

*Brisbane Case.* As regards the Brisbane case, the material was obtained by a colleague, Dr Bradley, so that portions likely to contain the virus were selected. The glycerinated material was necessarily kept at late summer temperature during transit, and was inoculated a week or ten days after the death of the patient.

*Conclusions.* Failure may be attributed to the elevated temperature during transit and the long period before the inoculation was made.



CASE 37. As regards the material from Case 37, A. C. F., Boggabri, the first monkey inoculated was *M. cynomolgus*. We have not so far obtained a successful result in the few inoculations we have made into this species of monkey. The patient died on the third day of the disease; the material was taken next day, and the monkey (A. 44, M. 3825) was inoculated on the succeeding day. A. 45, M. (*M. rhesus*) 3837, was inoculated twenty-nine days after the patient had died; that it was not immune to the virus was shown by a successful inoculation six weeks later (A. 72, M. 3873).

*Conclusions.* Whilst the failure of A. 45, M. 3837, to "take" might be attributed to the length of time that had elapsed after the patient had died, there seems no explanation, except perhaps the species of monkey employed, for the failure of A. 44, M. 3825. The material used in the glycerine emulsion was obtained from the frontal, parietal, occipital and temporo-sphenoidal regions of the cerebrum, and from the cerebellum, pons, medulla, and cervical, dorsal and lumbar regions of the cord— that is from parts which have been successfully employed in other cases. The child died on the third day of the disease before the virus could be expected to have died out. A post-mortem was made on the day after death— a period which allowed successful results in other cases. The first inoculation was made only two days after death— that is, after a period which gave success in other cases.

#### APPENDIX VI. *Failure in Monkeys of Certain Intracerebral Inoculations of Brain and Spinal Cord from Monkeys.*

A. 17, M. 3836, failed to develop the disease when inoculated on April 11th with material from A. 8, M. 3783, which material, when used on February 13th, conveyed the disease to A. 14, M. 3805. That A. 17 was not immune was shown by its successful inoculation later as A. 55, M. 3848. The inference is that the virus died out during its storage as a glycerine emulsion for two months.

A. 18 and A. 19. These two experiments are discussed under the sections dealing with the influence in monkeys of the treatment of the emulsion of the virus with various sera. Though the serum used in the case of A. 19 may have afforded protection, the "normal" human serum used in A. 18 cannot be expected to have done so in this case. The source of the supposed virus used in the experiments on A. 18 and A. 19 was A. 9, M. 3785, which had an unusually long incubation period of eleven to thirteen days, and a prolonged illness of ten or eight days. It is possible that, by the time this monkey was killed, the virus causing its disease had died out, or was only present in subinfective amount. This would explain the failure in both monkeys.

A. 20 and A. 21. The failures in these monkeys are easily explained, inasmuch as there was no histological evidence that the source of the virus, A. 11, M. 3801, had the disease at the time of its death.

A. 116. The failure in this monkey, when its fellow, A. 117, "took," is fully discussed in the section dealing with the influence of various sera on the virus. The evidence suggests that it was protected against infection by the serum of A. 89, Sh. 3902, which had had, and perhaps still had at the time of its death, the disease in question. Another explanation of the failure is that the strain employed had undergone a phase of weakening in virulence, through which the virus, on this account alone, failed to infect certain individual monkeys. Still another explanation is that the monkey was naturally immune, or had been rendered artificially immune by a previous intracerebral injection of material capable of causing immunity but not of producing the disease.

A. 132. This monkey is the same animal as A. 116. It failed to take when inoculated with material two days old from A. 129, M. 3967, which might reasonably have been expected to convey the disease. It is reasonable to suppose that artificial immunity had been

established in this monkey by the inoculation of the virus combined with a (presumed) immune serum, referred to under A. 116, or possibly by the first intracerebral inoculation.

*Summary.* Reasonable explanations are forthcoming for the failures of all these inoculations.

#### APPENDIX VII. *Failure of Intraperitoneal Inoculations.*

Intraperitoneal inoculations failed in animals as follows:

A. 2, M. (*Macacus rhesus*) 3777. Swabbings of the naso-pharynx were made from several contacts of Case 26, J. C. B., Narrabri, and the swabs were emulsified in a glycerine solution. The patient had died on January 15th, a day before these swabs were taken; the glycerine emulsion was inoculated on January 17th.

A. 6, M. (*M. rhesus*) 3781. From Case 27, A. B., Narrabri, dying on the third day of the disease. Post-mortem examination next day. Inoculation made two days after the patient's death with material from the brain and spinal cord.

A. 24, M. (*M. rhesus*) 3798. From Case 28, J. M., Narrabri, dying on the sixth day of the disease. Post-mortem examination next day. A glycerinated emulsion of the upper part of the cervical cord inoculated three days after the patient's death.

A. 67, Sh. 3861. From A. 65, Sh. 3855, dying with typical lesions of this disease. The glycerine emulsion of the brain and spinal cord inoculated two days after death.

*Analysis of Results.* From A. 2, M. 3777, inoculated from the swabbings of contacts of a case of the disease, nothing is to be learned.

The failure of A. 6, M. 3781, is important, inasmuch as A. 8, M. 3783, inoculated intracerebrally on the same day, and A. 9, M. 3785, and A. 10, M. 3786, inoculated intracerebrally on the next day—all took.

The failure of A. 24, M. 3798, must be considered in connection with the failures of A. 27, M. 3809, A. 28, M. 3810, and A. 29, M. 3819, injected intracerebrally. The period after death of the host at which the inoculation took place in this monkey was only two days. Even had infective material been present it is possible that, as in the case of A. 6, M. 3781, the monkey might not have taken.

A. 67, Sh. 3861, failed to take intraperitoneally, whilst the same material inoculated on the same day into A. 66, M. 3860, took intracerebrally. That this sheep was not naturally immune is shown by its successful inoculation six weeks later (A. 89, Sh. 3902).

*Conclusions.* A. 6, M. 3781 and A. 67, Sh. 3861, show that infective material injected intraperitoneally may fail to convey infection, whilst the same material injected intracerebrally may be successful.

#### APPENDIX VIII. *Failure of Intrasciatic Inoculations.*

A. 4, M. 3779, was inoculated in this way with an emulsion of the throat swabbings from the contacts of a case. The animal was *Macacus cynomolgus*—a species in which, in the few inoculations made into it, we have been so far unsuccessful in producing the disease. Further, we do not know as yet whether the virus is frequently present, or present at all, in the naso-pharynx of contacts or cases.

A. 5, M. 3780, was injected with an emulsion which "took" on the same date by intracerebral inoculation in the case of A. 8, M. 3783, and a day later by the same route in A. 9, M. 3785, and in A. 10, M. 3786.

#### APPENDIX IX. *Failure of Pasteur-Chamberland F. filtrates.*

All these inoculations were negative. To enable the material to pass through the filter it was necessary to dilute it to a considerable extent.

A. 26, M. 3807, need not be further considered inasmuch as the inoculation of other material from the same case failed to "take," and hence the presence of the virus in the material as used was not established.

A. 12, M. 3802, was inoculated with a filtrate from the ten times diluted emulsion of A. 8, M. 3783, on the day of this animal's death. As A. 14, M. 3805, inoculated next day with the same emulsion, diluted with an equal amount of monkey serum, developed the disease, the virus was manifestly present in the emulsion before its filtration.

A. 54, M. 3846, was inoculated with a filtrate obtained from an emulsion from A. 50, M. 3839, on the day of this animal's death. As A. 55, M. 3848, inoculated next day with the untreated emulsion, developed the disease, the virus was manifestly present in the original emulsion.

*Conclusion.* The diluted virus has been proved in two cases not to pass through the pores of a Pasteur-Chamberland F. filter, at least in sufficient quantity to produce infection in monkeys by the intracerebral injection of about 1 c.c. of filtrate.

#### APPENDIX X. *Failure of Cerebro-spinal Fluid to Cause Infection.*

A. 1, M. 3776, was unaffected by the intracerebral injection of cerebro-spinal fluid taken from a fatal case on the second day of illness and injected two days later.

A. 32, M. 3800, inoculated intracerebrally four days afterwards with cerebro-spinal fluid taken from a fatal case on the third day of illness, failed to take.

M. 3689, was inoculated intraperitoneally and intrathecally with cerebro-spinal fluid from Case 28, F. R., of the 1917 series.

#### APPENDIX XI. *Failure of a Pasteur-Chamberland F. Filtrate of Faeces.*

A. 31, M. 3779, received an intracerebral injection of a Pasteur-Chamberland F. filtrate of faeces obtained from Case 36, J. K., Boggabri. It remained unaffected. As failures have resulted from the use of such Pasteur-Chamberland F. filtrates obtained from material known to be virulent, the failure in this case teaches us nothing. Attention may also be called to the fact that the monkey used was *Macacus cynomolgus*, a species to which we have so far not been able to convey the disease.

*Summary.* The failure of this filtrate leaves open the question as to whether the virus may or may not be present in the faeces.

#### APPENDIX XII. *Failure of a "Noguchi Culture."*

An attempt was made to grow the virus from monkey material according to Noguchi's method for spirochaetes (*Jul. Exp. Med.* xvi, 1912, p. 621).

A diffuse cloud appeared in one of the original cultures which otherwise remained sterile, and this material was inoculated a month after the death of the monkey from which it was obtained. The animal remained perfectly well, and was later successfully inoculated with further material.

*Summary.* Failure resulted from the inoculation of a first generation of a presumed Noguchi culture a month after the death of the monkey from which it was made.

#### APPENDIX XIII. *Failure in Monkeys of Intracerebral Inoculations from the Nasopharyngeal Swabs of Contacts and of a Case.*

A. 3, M. 3778, was inoculated with an emulsion in glycerine solution of the nasopharyngeal swabs of several contacts of a case, and A. 30, M. 3797, with a similar swabbing from an actual case at the height of the disease. These experiments were conducted on the same animal, which happened to be *Macacus cynomolgus*, a species to which, in the few experiments we have made, we have so far been unsuccessful in conveying the disease. Apart from this, the method adopted was not one that entailed any concentration of the virus. These experiments, therefore, neither prove nor disprove the possible presence of the virus in the nasopharynx of contacts or cases.

APPENDIX XIV. *Failure in a Monkey of the Intracerebral Inoculation of Brain and Spinal Cord from a Horse.*

A. 42. This failure throws no light on the present disease. A horse died at Narrabri from a nervous complaint, which has not been shown to be connected in any way with human encephalo-myelitis.

APPENDIX XV. *Failure of Intracerebral Inoculation of an Emulsion of Fowl Ticks (*Argas persicus*).*

A. 43. The failure in this monkey throws no light upon the disease in question. The reason why an inoculation was made of an emulsion of fowl ticks is discussed in the section on the possibility of the occurrence of an intermediate (invertebrate) host of the virus in our full official report. It was thought possible that the human encephalo-myelitis might be due to some parasite transmitted by fowl ticks, such as the spirochaete producing the spirochaetosis of these birds.

The failure of the monkey does not support any such contention, though it does not necessarily exclude it.

APPENDIX XVI. *Failure in a Monkey and a Sheep after the Introduction of Horse Serum into the Spinal Canal and Introduction of the Virus into a Vein.*

The animals thus dealt with comprise A. 71, M. 3872, and A. 76, Sh. 3877. The virus in each instance was obtained from monkeys. In the case of A. 71, M. 3872, the virus had been kept for nine days, and, though no positive results were obtained from other inoculations of this virus, there is every reason to consider that it was present when the monkey yielding it was killed. In the case of A. 76, Sh. 3877, the virus was a day old, and had produced a successful result by intracerebral inoculation on the previous day in A. 72, M. 3873. The amount of the virus introduced, about 0.5 c.c., may have been too small to produce infection by this route in either animal. Further, as regards the monkey inoculation, the virus may have died out during the nine days in which it was kept in an ice-chest; and, as regards the sheep inoculation, the sheep employed is shown, under the designation A. 93, to have been naturally immune to the disease. These experiments, therefore, neither prove nor disprove the possibility of causing infection by the method employed.

APPENDIX XVII. *Table of Sheep, Calf and Horse Inoculations. Positive Results with Death.*

No.	Source of virus	Date of inoculation	Incubation period in days	Days of illness at death
A. 52, Sh. 3839 <i>b</i>	A. 49, M. 3835	16/4/1918	3	3
A. 65, Sh. 3855	A. 55, M. 3848	7/5/1918	6	3
A. 80, Sh. 3892	A. 72, M. 3873	17/6/1918	7	5
A. 81, Sh. 3893	A. 72, M. 3873	17/6/1918	12	4
A. 89, Sh. 3902	A. 78, M. 3890	27/6/1918	5 or 7	30 (death adventitious?)
A. 91, Sh. 3904	A. 78, M. 3890	27/6/1918	6	1
A. 92, Sh. 3905	A. 78, M. 3890	27/6/1918	4 (or more)	5 (or less)
A. 98, Sh. 3910	A. 92, Sh. 3905	6/7/1918	5	5
A. 103, Sh. 3915	A. 87, M. 3900	7/7/1918	9 (or 7)	2 (or 4)
A. 105, Sh. 3917	A. 87, M. 3900	7/7/1918	7 (or less)	3 (or more)
A. 108, Sh. 3920	A. 98, Sh. 3910	16/7/1918	6	1
A. 109, Sh. 3921	A. 103, Sh. 3915	18/7/1918	9 (possibly 6)	1 (possibly 4)
A. 121, Sh. 3941	A. 110, M. 3925	2/8/1918	5 or 7	7 or 5
A. 57, Calf 3848 <i>b</i>	A. 50, M. 3839	25/4/1918	5	3
A. 95, Horse 3908	A. 78, M. 3890	28/6/1918	9	3

APPENDIX XVIII. *Summary of Successful Inoculations in Sheep, a Calf and a Horse.*

The foregoing table (Appendix XVII) indicates the source of the virus (whether from a monkey or a sheep), the date of inoculation, the incubation period and the duration of the illness. In those cases in which previous inoculations had been made, details will be found in the section dealing with sheep-reinoculations.

A. 52, Sh. 3839 *b*, after an incubation period of three days, became slightly sick and seemed to have difficulty in getting its head down to nibble grass. Next day the animal was restless; its legs seemed somewhat rigid; there was stiffness in the neck, and the head was turned to one side; the lower jaw quivered, the ears twitched and the animal tended to walk in a circle, and took fits during which the head was thrown back and it pawed the air. It presented much the same symptoms on the next day and was unconscious on the third day of illness when it died-- exactly six days after the inoculation.

A. 65, Sh. 3855, became ill after an incubation period of six days. The symptoms consisted of shallow and fairly rapid respirations, some stiffness of the neck, quivering of the nostrils and a somewhat slow gait. On the second day it was very sick, would jump round, fall on the ground and struggle, and had convulsive movements. It died on the morning of the third day of illness.

A. 80, Sh. 3892, after an incubation period of seven days, became ill, holding its head down and lying down most of the time. Next day its lips were quivering and the head was turned to one side. On the fourth day it was dribbling from the mouth; there was no apparent paresis. On the fifth day it was lying on its side with the head thrown slightly back and had frequent convulsive movements. At this stage it was killed.

A. 81, Sh. 3893, became ill on the twelfth day. It ran holding its head high and breathing quickly. The ears and lips were twitching slightly. Its temperature was 106° F. Next day, in addition, the animal tended to circle round holding its head high. On the fourth day it was lying on its side with the head somewhat retracted. It manifested convulsive movements from time to time and twitched all over.

A. 89, Sh. 3902, showed, on the fifth day after inoculation, rapidity of breathing, protrusion of the tongue, and running from the nose. Next day it was still breathing quickly. On the seventh day it fell down on one occasion and struggled, and later held its head high and made "champing" movements of the head, lips and jaws. On the eighth day it seemed well again. Twenty-one days after the inoculation it was bled, and it was bled again thirteen days later. Two days after the last bleeding it apparently had respiratory trouble, became worse next day, and died thirty-eight days after the inoculation. Typical histological changes were present.

A. 91, Sh. 3904, on the second and third days (excluding the day of inoculation) seemed ill, the symptoms suggesting some respiratory trouble following the anaesthetic. On the fourth and fifth days it was better. On the sixth day it was not feeding; it turned slowly in a circle and then fell on its side with retraction of the head and trembling of the lips and nostrils. Convulsions then supervened and the animal seemed unconscious. The respirations were highly irregular. It died an hour after the manifestation of these grave signs.

A. 92, Sh. 3905, on the fourth to the sixth day after inoculation seemed ill, and on the last of these days had a mucous discharge from the nose. On the seventh day its temperature was 105° F. and the respirations were sometimes rapid. When placed on its legs it moved backwards and to one side with a stiff and jerky gait. It was lying down most of the time with the ears twitching. Next day it was much the same. There was a mucous discharge

from the nostrils and very irregular respirations, and occasionally convulsive movements. The temperature reached 106° F. It died early next day.

A. 98, Sh. 3910, became ill on the fifth day. On the sixth day the temperature was 107.4° F. It was drowsy and the breathing was rapid. On the eighth day it tended to circle towards one side and ran into objects, and was breathing rapidly. On the ninth day it showed convulsive movements all day, and died during the night.

A. 103, Sh. 3915, had a temperature of 107° F. on the sixth day but did not show definite symptoms until the ninth day. Its temperature now was 104°. It showed occasional twitchings and was breathing rapidly, and died on the tenth day.

A. 105, Sh. 3917, had a temperature of 106° F. on the fifth day. On the seventh day it was dribbling from the mouth and hardly moved. On the eighth day it had a convulsion and there seemed to be weakness in the hind legs. On the ninth day there were continuous twitchings of the nose and mouth, and it died during the following night.

A. 108, Sh. 3920, six days after inoculation, became sick and had rapid respirations. The same evening it exhibited "staggers," and died during the night.

A. 109, Sh. 3921, between the second and eighth days showed occasionally rapid breathing, but no other definite symptoms. It became definitely ill on the ninth day. The head was retracted, the animal circled and showed twitchings of the lips. These were followed by convulsive movements and death.

A. 121, Sh. 3941, on the fifth day after inoculation, was breathing fast. Next day it was not taking its food and seemed weak and the temperature was raised. Convulsive movements appeared on the seventh day after inoculation and occurred again the following day. On the ninth day it had intense convulsive movements lasting for about an hour, together with fine quiverings of the ears and eyelids, twitchings of the head and working movements of the jaws, and very irregular breathing. The animal seemed unconscious during the convulsive attacks. When these had passed off the animal could only stand by leaning against some support, and in walking it swayed from side to side dragging one hind leg a little. Next day the head was retracted and the back markedly arched. Respirations were irregular and there was continual grinding of the teeth. On the eleventh day after inoculation it was lying on its side. The head was slightly retracted; the tail and back legs moved on stimulation. The animal died in the afternoon.

A. 57, Calf 3848 *b*, first showed symptoms five days after inoculation, when it kept its head dependent, inclined to "go" in the front legs, and was restless. Next day there was a clear discharge from the nostrils. On the third day of illness the animal was walking about, the legs were weak and the gait tended to be circular. General convulsive seizures then developed, accompanied by rigidity of the limbs and muscular tremors, whilst the animal lay on its side with the head markedly retracted and the back arched. It was possibly unconscious. After the convulsions had continued for about twenty minutes the animal became fairly quiet, but died an hour later. Typical lesions were found on histological examination.

A. 95, Horse 3908, on the ninth day after inoculation had two seizures, during which it walked round towards the left. Next day its head was depressed, and on moving it tended to "go" towards the left side and threatened to fall on this side. There were twitchings of the facial muscles, staring eyes and apparently partial blindness. On the third day (of illness) the animal was lying on its right side with the head drawn to the left and manifested irregular movements of the left fore and hind limbs. The nostrils were working and the mouth at times was drawn to the left side. Later, intense and repeated convulsions developed alternating with short quiescent periods, and at this stage the animal was killed. Histological examination showed typical lesions.

**APPENDIX XIX. *Sheep showing no Symptoms after an Intracerebral Inoculation of Brain and Spinal Cord from Human Cases.***

A. 35. On February 26th this sheep was inoculated intracerebrally with material from a human case which infected A. 33, M. 3803, when similarly inoculated on February 13th. Later this sheep, under the designation A. 65, was successfully inoculated from a monkey. This shows that the failure in the first inoculation was not due to natural immunity, and the inference therefore is that the virus had died out during the thirteen days of its storage in an ice-chest between the time of the successful inoculation of the monkey and its use on this sheep.

A. 46. This happens to be the same sheep as A. 35, and as is thereunder indicated it was finally successfully inoculated under the designation A. 65, so that it clearly possessed no natural immunity to the disease. The first inoculation was made on April 16th with material from Case 37, A. C. P. (J.). As previous inoculations of this material on March 15th and April 11th into monkeys were both failures there is no evidence that the virus was present in the materials used at the time of any of these inoculations.

*Comment.* There are reasonable explanations for these failures.

**APPENDIX XX. *Sheep Showing no Symptoms after the Intracerebral Inoculation of Brain and Spinal Cord from Infected Monkeys.***

A. 53 and A. 84. The failure in these experiments is discussed in the section dealing with reinoculations. In these animals later inoculations were successful, and reasonable explanations are given for the initial failures.

A. 63 (93), A. 69 (94), A. 83, A. 101, A. 104. These experiments are also discussed in the section dealing with reinoculations. The evidence indicates that these sheep were naturally immune to the disease.

A. 75. Death occurred on the fifth day, apparently from lung trouble. No histological lesions of encephalo-myelitis could be detected though convulsive movements on the fourth day suggested the possibility of a "take." As the incubation period in some sheep has been apparently as short as three days, it is possible that this animal really did have the disease, but died before recognisable histological changes developed.

A. 96. The inoculation in this sheep was made with material preserved for over a month in glycerine in an ice-chest and obtained from a monkey. In the fresh state, this virus conveyed the disease to a monkey, a sheep and a horse. Failure may be attributed either to the length of time the material was preserved or to natural immunity.

A. 106. This animal died from post-anæsthetic lung trouble five to six days after inoculation. No evidence of the disease was detected histologically, though it might or might not have had time to develop. This sheep may have been naturally immune.

A. 111. Since the same material, as was used in this experiment, was successful on the same day in conveying the disease to A. 110, M. 3925, the failure is to be attributed to a natural immunity.

A. 122. The successful results in A. 117, M. 3937, and in A. 121, Sh. 3941, on the same date with the same material, indicate that natural immunity is the explanation of the failure in A. 122.

*Summary.* In seven of these sheep, natural immunity seems clearly to be the explanation of the failures. In two, death occurred so early, from complications, that the disease, if about to develop, may not have had time to manifest itself or produce recognisable lesions. In one, the length of storage of the virus may have destroyed its activity. In the other two, reasonable explanation of the failures are given elsewhere.

APPENDIX XXI. *Sheep showing no Symptoms after the Intracerebral Inoculation of Brain and Spinal Cord from Infected Sheep.*

A. 68, A. 114, and A. 115. These three animals are all discussed in the section dealing with re-inoculations, where it is shown that they were naturally immune.

A. 126. This animal failed to take definitely whilst its fellow, A. 125, showed symptoms, possibly due to encephalo-myelitis, and recovered. A. 126 may have enjoyed natural immunity.

APPENDIX XXII. *Failure in a Sheep of an Intracerebral Inoculation of Dried Brain and Spinal Cord from a Positive Monkey.*

A. 77, Sh. 3878, received an intracerebral injection of emulsified dried brain and spinal cord from a positive monkey eight days after this monkey's death. The material had been dried in Petri dishes in an incubator.

As many sheep are immune to intracerebral inoculation of the virus, the failure in this case neither proves nor disproves the possibility of the virus resisting desiccation for eight days at incubator temperature.

APPENDIX XXIII. *Failure in Sheep of a Berkefeld Filtrate from a Positive Monkey.*

A. 119, Sh. 3939, and A. 120, Sh. 3940, received intracerebral injections of a Berkefeld filtrate and remained unaffected, whilst inoculations on the same date of the unfiltered emulsion were successful in conveying the disease to A. 117, M. 3937, and A. 121, Sh. 3941.

One of these sheep, A. 119, Sh. 3939, received a further inoculation twenty-six days later, which was followed a week later by symptoms suggesting slight encephalo-myelitis from which the animal recovered. There is reason therefore to think that this sheep was not naturally immune.

The inference to be drawn from these experiments is that the virus did not pass through the pores of the Berkefeld filter, at least in sufficient quantity to induce infection.

APPENDIX XXIV. *Failure to Convey the Disease to a Sheep by Intranasal Swabbing.*

A. 70, Sh. 3865, had the inside of the nostrils vigorously swabbed with an emulsion of the brain and spinal cord of A. 64, M. 3854 and was unaffected thereby. A. 69, Sh. 3864, inoculated intracerebrally with the same material, on the same date, failed to develop the disease. A. 71, M. 3872, seven days later had an injection of horse's serum into the spinal canal and a small quantity of the material from this monkey (A. 64) introduced into a vein. It also remained well. These were the only three animals receiving inoculations from A. 64, M. 3854, and none of them developed the disease. Only one was inoculated intracerebrally, the only route by which we have so far obtained infections, and the animal so inoculated was a sheep, a species which we have found frequently to be immune to the virus introduced by this route. That A. 70 was not immune to the virus was shown later, under the designation A. 92, Sh. 3905, when the disease was successfully conveyed to it.

*Comment.* The result of this experiment neither proves nor disproves the possibility of infection occurring through the nose.

APPENDIX XXV. *Sheep Reinoculations.*

For various reasons—economy of expensive animals and testing for natural immunity being the most important—a number of sheep were reinoculated. The animals so dealt with were fourteen in number. They may be divided into those which "took" after reinoculation



and those in which reinoculations were unsuccessful. Unless otherwise stated, the inoculation was intracerebral and the material used an emulsion of portions of the brain and spinal cord. There were six in the first and eight in the second group.

(a) *Successful Reinoculations.*

A. 23, Sh. 3816—A. 52, Sh. 3839 *b*. The first inoculation was made on February 26th with material from A. 11, M. 3801. It was afterwards found by histological examination that this monkey did not show the lesions of encephalo-myelitis. The later successful result by the inoculation on April 16th can therefore be explained.

A. 35, Sh. 3817—A. 46, Sh. 3839 *c*—A. 65, Sh. 3855. The first inoculation was made on February 26th with material from Case 38, C. H. From this human case A. 33, M. 3803, had been successfully inoculated on February 13th. The second inoculation was made on April 16th with material from Case 37, A. C. F. (J.); A. 44, M. 3825, was unsuccessfully inoculated on March 15th and A. 45, M. 3837, on April 11th with the same material. The successful result of the inoculation on May 7th from a monkey, A. 55, M. 3848, may be explained in connection with the first inoculation by the view that the glycerinated virus may have died out in the interval of a fortnight that elapsed between the successful inoculation in the monkey and the unsuccessful one in this sheep. As regards the second inoculation there was no evidence by the two monkey inoculations that the virus was present in the material used.

A. 53, Sh. 3863—A. 91, Sh. 3904. The first inoculation was made with material which, though originally infective, as shown by positive results in A. 50, M. 3839, and A. 52, Sh. 3839 *b*, had been conserved in the ice-chest for a month. The later positive result is therefore best explained by supposing that the first virus had died out during the period of its storage.

A. 67, Sh. 3861—A. 77, Sh. 3878—A. 89, Sh. 3902. The first inoculation on May 17th was an intraperitoneal one of an emulsion from a positive sheep, A. 65, Sh. 3855. The second experiment was on June 7th, and consisted of the intracerebral injection of dry brain material from A. 66, M. 3860. The third inoculation was made on June 27th from A. 78, M. 3890. A few days after the last inoculation it seemed sick and presented a few slight symptoms, possibly indicating a mild form of the disease. On July 18th blood was taken under an anaesthetic and it was bled again similarly on July 31st. It died on August 4th, apparently from lung trouble as a result of the anaesthetic, but histological examination showed the lesions of encephalo-myelitis, possibly in an early stage of resolution. Serum from the blood taken on the first occasion may have protected A. 111, Sh. 3922. It certainly seems as though the second sample of blood, when mixed with the virus before injection, protected A. 116, M. 3936.

In connection with the first injection, which was into the peritoneum, we possess as yet no evidence that this is a successful route for introducing the virus. As regards the second inoculation which was with dry brain material, the drying may reasonably be considered as having destroyed the virus. This animal cannot therefore be considered to have been proved to be naturally immune to the disease before the third inoculation with its delayed successful result.

A. 70, Sh. 3865—A. 92, Sh. 3905. The first experiment, performed on May 20th, consisted in swabbing the nose with an emulsion from A. 64, M. 3854. The successful result, therefore, of the intracerebral inoculation on June 27th can be explained by the first procedure not having been a reliable method for obtaining infection.

A. 84, Sh. 3896—A. 109, Sh. 3921. The first inoculation was made with tissues from the frontal and occipital regions only of the monkey yielding the virus. The general emulsion of the brain and spinal cord of this monkey gave positive results in A. 78, M. 3890; A. 80, Sh. 3892; and A. 81, Sh. 3893. The second inoculation, which was successful, was with the

mixed emulsion of brain and spinal cord from an infected sheep. The failure of the first inoculation may be explained by absence, relative or complete, of virus in the material used.

(b) *Unsuccessful Reinoculations.*

A. 63, Sh. 3853—A. 76, Sh. 3877—A. 93, Sh. 3906—A. 115, Sh. 3934. The first inoculation was made on May 3rd from a positive monkey, A. 62, M. 3845. The second experiment, performed on May 31st, consisted of lumbar puncture with the intraspinal injection of 2 c.c. of antimeningoococcal serum, together with an injection into a vein of 0.5 c.c. of virus from A. 66, M. 3860. The third experiment, on June 27th, consisted of the intracerebral injection of a saline emulsion of the medulla of A. 78, M. 3890. The companion sheep, A. 92, Sh. 3905, similarly injected at the same time, gave a positive result. The fourth inoculation was made on July 29th from a positive case in a sheep, A. 109, Sh. 3921. It is clear that the material used for the third inoculation contained the virus at the time it was used, while it is almost certain that the virus was also present in the material used for the first and fourth inoculations though there were no control animals to prove this conclusively. The technique of the second operation cannot be considered, as yet, a proved method of obtaining infection. These results suggest that the sheep has a natural immunity to the virus, which even the actual introduction of the virus into the brain cannot break down.

A. 68, Sh. 3862—A. 86, Sh. 3879—A. 90, Sh. 3903. The first inoculation was on May 17th with material from a positive sheep, A. 65, Sh. 3855. The second inoculation was on June 7th with material from A. 75, Sh. 3876. A. 75, Sh. 3876, died four days after inoculation and histological examination of its brain and spinal cord was negative. The third inoculation was on June 27th with virus from A. 78, M. 3890, which "took" in the companion sheep A. 89, Sh. 3902; A. 91, Sh. 3904; and A. 92, Sh. 3905. As regards the first inoculation we know from A. 66, M. 3860, that the virus was present on the date of inoculation. We also know, as indicated above, that the virus was present in the material used for the third inoculation. This animal would seem, therefore, to have possessed a natural immunity to the disease.

A. 69, Sh. 3864—A. 94, Sh. 3907. The first inoculation was made on May 20th with material from A. 64, M. 3854. The second inoculation was made on June 27th and consisted of an emulsion of the frontal and occipital regions of the brain of A. 78, M. 3890. As regards the first inoculation we have no proof—by means of a control successfully-inoculated animal—that the virus was present in the material used, but inasmuch as this monkey presented the typical histological appearances of the disease, there is every reason to suppose that the virus was present. As regards the second inoculation, we have no proof that the virus is consistently present in the frontal and occipital areas of the brain, though histological examinations of affected animals would suggest that it is so, at least frequently. This sheep therefore also seems to have possessed a natural immunity.

A. 83, Sh. 3895—A. 114, Sh. 3933. The first inoculation was made on June 17th with material from A. 72, M. 3873, companion sheep, namely, A. 80, Sh. 3892 and A. 81, Sh. 3893, giving positive results. The second inoculation was made on July 29th from a positive sheep, A. 109, Sh. 3921. A companion animal of this last inoculation, which was also a previously inoculated animal, likewise remained unaffected, so that there was no control successful case for the second inoculation. The results of these two inoculations seem to show again a natural immunity in this animal.

A. 101, Sh. 3913—A. 125, Sh. 3948. The first inoculation was made with material which successfully conveyed the disease to A. 100, M. 3912; to A. 103, Sh. 3915; to A. 105, Sh. 3917; and probably to A. 102, Sh. 3914, which recovered. The virus was therefore evidently present in the material used on A. 101, Sh. 3913. The second inoculation was with material obtained from a positive sheep, A. 121, Sh. 3941. Decomposition was commencing in the carcass when the tissues were obtained for inoculation purposes. On the fourth and fifth days the inocu-

lated animal showed slight symptoms suggestive of encephalo-myelitis but recovered. Inoculations of the same material on the same day into another sheep, a calf, and a monkey (*Macacus cynomolgus*) were negative. The results of these experiments again suggest a natural immunity, not perhaps complete or varying from time to time in degree.

A. 104, Sh. 3916—A. 126, Sh. 3949. The same remarks apply to A. 104, Sh. 3916, regarding the first inoculation, as to the above-mentioned A. 101, Sh. 3913, the successful experiment in A. 103, Sh. 3915, being an exact counterpart. The second inoculation was also with the same material as was used on the above A. 125, Sh. 3948, but no illness resulted. Natural immunity to the virus, as evidenced by the first inoculation, seems to be again evident in this animal.

A. 119, Sh. 3939—A. 130, Sh. 3968. The first inoculation was with a Berkefeld filtrate of material which, unfiltered, conveyed the disease to A. 117, M. 3937, and to A. 121, Sh. 3941. After the second inoculation, with monkey material that successfully conveyed the disease to A. 129, M. 3967, an illness, probably encephalo-myelitis, developed, from which the sheep recovered. The inference drawn from these experiments is that the virus was not present, or was only present in subinfective amount, in the filtrate used.

A. 120, Sh. 3940—A. 131, Sh. 3969. These were parallel inoculations to those in the previous case. A possible mild attack of encephalo-myelitis followed the second inoculation. The same comment applies.

*Inferences drawn.* As regards the six sheep in which the final inoculations were successful, there seem to be valid reasons why the first were unsuccessful.

As regards the unsuccessful reinoculations, in the cases of the first three sheep the presence of a natural immunity to the introduction of the virus into the brain seems to be reasonably established. As regards the fourth sheep, there appears to be evidence of a natural immunity, perhaps not complete or varying in degree from time to time. The last two sheep suggest that the virus is held back, completely or to a large degree, by the pores of a Berkefeld filter.

#### APPENDIX XXVI. (a) *Calves showing Symptoms of Illness, possibly due to Encephalo-myelitis, after Intracerebral Inoculation of Material from the Brain and Spinal Cord of a Positive Monkey or a Positive Horse.*

A. 88. On June 27th this calf was inoculated intracerebrally with material from a monkey which successfully conveyed the disease on the same date to A. 87, M. 3900; A. 89, Sh. 3902; A. 91, Sh. 3904; A. 92, Sh. 3905; and A. 95, Horse 3908.

On July 1st the calf was sick and staggered, being especially weak in the hind legs. In the afternoon shivers were noticed in the hindquarters. On July 2nd it was very sick. It kept its head down, tended to walk in a circle, was very jerky on its legs, and stiff in the hindquarters and drowsy. In the evening it had convulsive movements, during which it bellowed, jumped in the air, fell down and struggled, and appeared to be unconscious. On July 3rd its hind legs swayed on movement, and it seemed weak and fell down at times. Next day it was better and could get up by itself from the ground, and was apparently almost normal in behaviour. On July 5th it could walk about, but swayed slightly on movement. On July 7th it was in much the same condition, though it had occasional convulsive seizures, and was not inclined to "play." It swayed a little on movement and there were occasional twitchings in one hind leg. On July 12th it was lying down and seemed drowsy. On July 14th it developed diarrhoea, which continued for about a fortnight, but from which it eventually recovered.

Considering the success of the material used in other animals, and the nature of the symptoms manifested, there seems little doubt that this calf had a mild form of encephalo-myelitis, from which it recovered.

A. 107. On July 10th this calf was inoculated intracerebrally with material from A. 95, the successfully inoculated horse. It seemed ill two days later, and on July 13th seemed very

sick. On July 14th it was weak and "wobbly" on the legs but otherwise normal. On July 16th it was better and thereafter showed no signs of illness until August 14th, when it received another inoculation (*vide infra* A. 127).

It is possible that the slight symptoms shown were due to a mild attack of encephalomyelitis from which the animal recovered.

(b) *Calf showing no Symptoms after Intracerebral Inoculation (a second inoculation) of Brain and Spinal Cord from a Positive Sheep.*

A. 127. This calf is the same animal as has just been referred to under the designation A. 107. The second inoculation was made on August 14th and the calf showed no symptoms which could be attributed to encephalo-myelitis as the result of this inoculation.

It is possible that the first inoculation had rendered the animal immune to the later introduction of virus.

#### APPENDIX XXVII. *Failure to Convey the Disease to Dogs.*

Inoculation into A. 34, Dog 3806, failed on February 14th, while a monkey, A. 33, M. 3803, inoculated the day before with the same material developed the disease.

A. 16, Dog 3813, was inoculated on February 21st with material from A. 8, M. 3783, and failed to take, whilst A. 14, M. 3805, inoculated on February 13th with the same material developed the disease. During the eight days that elapsed between these inoculations, the virus may possibly have died out.

A. 56, Dog 3847, was inoculated from A. 50, M. 3839, unsuccessfully, whilst A. 55, M. 3848, inoculated on the same day with the same material developed the disease. This dog was the same animal as A. 16.

A. 97, Dog 3909. This was a third attempt to inoculate the animal shown as A. 16 and A. 56. This time fresh material from a positive sheep was used for the intracerebral inoculation. The result was again negative.

*Comment.* It is clear that in three of these inoculations, if not in all, the material used contained the virus. The dog has therefore not been shown to be susceptible to the disease.

#### APPENDIX XXVIII. *Failure to Convey the Disease to a Kitten by Intracerebral Inoculation.*

A. 79, Kitten 3891, received an intracerebral inoculation of an emulsion of brain and spinal cord from A. 72, M. 3873, and remained perfectly well afterwards, whereas A. 78, M. 3890, inoculated on the same day with the same material developed the disease.

#### APPENDIX XXIX. *Failure to Convey the Disease to Rabbits.*

A. 58, Rabbit 3849, was inoculated from A. 50, M. 3839, and failed to take. A. 55, M. 3848, inoculated with the same material, the day before, developed the disease.

A. 73, Rabbit 3874, inoculated from A. 66, M. 3860, failed to take, whilst A. 72, M. 3873, inoculated on the same day, developed the disease.

*Summary.* Two rabbits inoculated with material shown to contain the virus did not contract the disease.

#### APPENDIX XXX. *Doubtful Results in Guinea-pig Inoculations.*

Two guinea-pigs, A. 60, Gp. 3851, and A. 74, Gp. 3875, were inoculated. In the case of the first the material was obtained from a monkey the day after the same material conveyed the disease to A. 55, M. 3848, and A. 57, Calf 3848*b*. In the case of the second guinea-pig, the material used gave a positive result when inoculated on the same day into A. 72, M. 3873.

A. 60, Gp. 3851, six days after the inoculation was very sluggish and its hind legs seemed slightly weak, and on the following day it hardly moved at all. It died on the third day of illness. Histological examination showed some slight changes which might or might not be interpreted as evidence of a very mild form of encephalo-myelitis. The second guinea-pig, A. 74, Gp. 3875, remained apparently well until nearly a month after the inoculation, when its head was somewhat retracted and it could not raise itself up or walk. It died during the first day of this illness. Histological examination again showed some slight changes though these were probably not due to encephalo-myelitis.

*Summary.* One guinea-pig showed symptoms and histological lesions which might or might not be attributable to a very mild form of encephalo-myelitis. The second guinea-pig probably gave a negative result.

#### APPENDIX XXXI. *Failure to Convey the Disease to a Hen by Intracerebral Inoculation.*

A. 118, Hen 3938, received an intracerebral inoculation of material from A. 110, M. 3925, and remained perfectly unaffected whilst a monkey and a sheep inoculated on the same day with the same material developed the disease. This inoculation was specially made into a hen on account of the possibility of the human disease being the same as the spirochaetosis of fowls so common in the affected districts.

*Summary.* Virulent material failed to convey the disease to a hen by intracerebral inoculation.

#### APPENDIX XXXII. *Table showing the various Animal Inoculations.*

(Unless otherwise stated, the inoculations were made intracerebrally and the material comprised portions of the cortex of the cerebrum, pons, medulla, and spinal cord. + means a successful result, - means an unsuccessful result, and a blank indicates that the inoculated animal died too soon to allow manifestations of the disease to appear, supposing the virus had been transmitted. M = monkey, Sh = sheep, m. = inoculated, d. = died, k. = killed. Numbers in brackets [e.g. (A. 33)] mean that the animal so referred to received other inoculations under such designations. Roman figures represent generations.)

##### CASE 26. J. C. B., Narrabri.

(Ill four days; cerebro-spinal fluid taken 14/1/1918; swabbings of contacts taken 16/1/1918; patient died 15/1/1918.)

- A. 1 (A. 33), M. 3776 ( - ), cerebro-spinal fluid (in. 15/1/18).
- A. 2 (A. 13), M. 3777 ( - ), nasopharyngeal swabs, intraperitoneal (in. 17/1/18).
- A. 3 (A. 36, A. 44, A. 51), M. 3778 ( - ) nasopharyngeal swabs (in. 21/1/18).
- A. 4 (A. 22, A. 31), M. 3779 ( - ), nasopharyngeal swabs, into sciatic nerve (in. 22/1/18)

##### CASE 27, A. B., Narrabri.

- A. 5 (A. 21), M. 3780 ( - ), into sciatic nerve (in. 29/1/18).
- A. 6 (A. 20), M. 3781 ( - ), intraperitoneal (in. 29/1/18).
- A. 7, M. 3782
- A. 8, M. 3783 ( + ), (in. 29/1/18; ill 7/2/18; k. 12/2/18).
- A. 9, M. 3785 ( + ), (in. 30/1/18; ill 11/2/18; k. 21/2/18).
- A. 10, M. 3786 ( + ), (in. 30/1/18; ill 14/2/18; k. 16/2/18).

From A. 8, M. 3783, (in. 29/1/18; k. 12/2/18).

- II. A. 11, M. 3801 ( - ), (in. 12/2/18; d. with milary abscesses 25/2/18)
- A. 12, M. 3802 ( - ), Pasteur-Chamberland F. filtrate (in. 12/2/18).
- A. 13 (A. 2), M. 3804.
- A. 14, M. 3805 ( + ), (in. 13/2/18; ill 22/2/18; d. 25/2/18).

A. 15, Dog 3808.

A. 16, Dog 3813 ( - ), (in. 21/2/18).

A. 17 (A. 64), M. 3836 ( - ), material kept two months (in. 11/4/18).

*From* A. 9, M. 3785, (in. 30/1/18; k. 21/2/18).

A. 18 (A. 27), M. 3823 ( - ), (in. 15/3/18).

A. 19 (A. 26), M. 3824 ( - ), (in. 15/3/18).

*From* A. 11, M. 3801, (in. 12/2/18; d. from miliary abscesses 25/2/18)

A. 20 (A. 6), M. 3814 ( - ), (in. 27/2/18).

A. 21 (A. 5), M. 3815 ( - ), (in. 27/2/18).

A. 22 (A. 4, A. 31), M. 3818.

A. 23 (A. 52), Sh. 3816 ( - ), (in. 26/2/18).

#### CASE 28, J. M., Narrabri.

(Ill six days; died 6/2/18; cervical cord and adjacent part of brain only used.)

A. 24 (A. 29), M. 3798 ( - ), intraperitoneal (in. 9/2/18).

A. 25, M. 3799 (abscess in five days).

A. 26 (A. 19), M. 3807 ( - ), Pasteur-Chamberland F. filtrate (in. 15/2/18).

A. 27 (A. 18), M. 3809 ( - ), treated with serum (in. 16/2/18).

A. 28 (A. 43, A. 55 + ), M. 3810 ( - ), treated with serum (in. 16/2/18).

A. 29 (A. 24), M. 3819 ( - ), (in. 6/3/18).

#### CASE 36, J. K., Boggabri.

(Ill five days; died 8/2/18.)

A. 30 (A. 3, A. 44, A. 51), M. 3797 ( - ), nasopharyngeal swab (in. 9/2/18).

A. 31 (A. 4, A. 22), M. 3779 ( - ), filtrate of faeces (in. 9/2/18).

A. 32 (A. 41, A. 42), M. 3800 ( - ), cerebro spinal fluid (in. 11/2/18).

#### CASE 38, G. H., Wee Waa.

(Ill two days; died midnight 11-12/2/18.)

A. 33 (A. 1), M. 3803 ( + ), (in. 13/2/18; ill 25/2/18; k. 2/3/18).

A. 34, Dog 3806 ( - ), (in. 14/2/18).

A. 35 (A. 46, A. 65 + ), Sh. 3817 ( - ), (in. 26/2/18).

*From* A. 33, M. 3803 (k. 2/3/18).

II. A. 36 (A. 62 + ), M. 3834 ( - ), Noguchi culture (in. 4/4/18).

#### BROKEN HILL CASES.

A. 37 (A. 39, A. 66 + ), M. 3811 ( - ), case 6; patient ill five days; d. 13/2/18 (in. 21/2/18).

A. 38 (A. 47), M. 3812 ( - ), case 7; patient ill four days; d. 13/2/18 (in. 21/2/18).

A. 39 (A. 37, A. 66 + ), M. 3830 ( - ), case 18; patient ill eight days; d. 16/3/18 (in. 27/3/18).

#### BRISBANE CASE.

A. 40, M. 3828 ( - ), patient d. about ten days previously (in. 22/3/18).

#### NARRANDERA CASE.

A. 41 (A. 32, A. 42), M. 3831 ( - ), case 54, G.S.; ill twelve days; d. 22/2/18 (in. 27/2/18).

#### HORSE.

A. 42 (A. 32, A. 41), M. 3820 ( - ), horse d. 27/2/18 (in. 6/3/18).

#### FOWL TICKS, from Boggabri.

A. 43 (A. 28, A. 55 + ), M. 3827 ( - ), (in. 16/3/18).

(CASE 37, A. C. F. (J.), Boggabri.

(Ill three days; died 13/3/18.)

A 44 (A. 3, A. 30, A. 51), M. 3825 ( - ), (in. 15/3/18).

A. 45 (A. 72 + ), M. 3837 ( - ), (in. 11/4/18).

A. 46 (A. 35, A. 65 + ), Sh. 3839 c ( - ), (in. 16/4/18).

(CASE 32, L. B., Narrabri.

(Ill five days; died 15/3/18.)

I. A. 47 (A. 38), M. 3826.

A. 48, M. 3829 ( + ), (in. 22/3/18; ill 2/4/18; k. 4/4/18).

*From* A. 48, M. 3829 (k. 4/4/18).

II. A. 49, M. 3835 ( + ), (in. 4/4/18; ill 11/4/18; k. 15/4/18).

*From* A. 49, M. 3835 (k. 15/4/18).

III. A. 50, M. 3839 ( + ), (in. 15/4/18; ill 20/4/18; k. 24/4/18).

A. 51 (A. 3, A. 30, A. 44), M. 3840.

A. 52 (A. 23), Sh. 3839 b ( + ), (in. 16/4/18; ill 19/4/18; d. 22/4/18).

A. 53 (A. 91 + ), Sh. 3863 ( - ), (in. 17/5/18)

*From* A. 50, M. 3839 (k. 24/4/18).

IV. A. 54 (A. 71), M. 3846 ( - ), Pasteur-Chamberland F. filtrate (in. 24/4/18).

A. 55 (A. 28, A. 43), M. 3848 ( + ), (in. 25/4/18; ill 3/5/18; d. 5/5/18).

A. 56 (A. 16, A. 97), Dog 3847 ( - ), (in. 25/4/18).

A. 57, Calf 3848 b ( + ), (in. 25/4/18; ill 30/4/18; d. 2/5/18)

A. 58, Rabbit 3849 ( - ), (in. 26/4/18).

A. 59, Rabbit 3850

A. 60, G. pig 3851 (?), (in. 26/4/18; ill 2/5/18; d. 4/5/18).

A. 61, Kitten 3852.

*From* A. 52, Sh. 3839 b (d. 22/4/18).

A. 62 (A. 36), M. 3845 ( + ), (in. 23/4/18; ill 29/4/18; k. 3/5/18).

*From* A. 62, M. 3845 (k. 3/5/18).

V. A. 63 (A. 76, A. 93, A. 115), Sh. 3853 ( - ), (in. 3/5/18).

*From* A. 55, M. 3848 (d. 5/5/18).

A. 64, M. 3854 ( + ), (in. 7/5/18; ill 14/5/18; k. 18/5/18).

A. 65 (A. 35, A. 46), Sh. 3855 ( + ), (in. 7/5/18; ill 13/5/18; d. 14 15/5/18).

*From* A. 65, Sh. 3855 (d. 14-15/5/18).

VI. A. 66 (A. 37, A. 39), M. 3860 ( + ), (in. 17/5/18; ill 22/5/18; k. 30/5/18).

A. 67 (A. 77, A. 89), Sh. 3861 ( - ), intraperitoneal (in. 17/5/18).

A. 68 (A. 86, A. 90), Sh. 3862 ( - ), (in. 17/5/18)

*From* A. 64, M. 3854 (k. 18/5/18).

A. 69 (A. 94), Sh. 3864 ( - ), (in. 20/5/18).

A. 70 (A. 92 + ), Sh. 3865 ( - ), nose swabbed (in. 20/5/18).

A. 71 (A. 54), M. 3872 ( - ), lumbar puncture, venous injection (in. 27/5/18).

*From* A. 66, M. 3860 (k. 30/5/18).

VII. A. 72 (A. 45), M. 3873 ( + ), (in. 30/5/18; ill 13/6/18; k. 15/6/18).

A. 73, Rabbit 3874 ( - ), (in. 30/5/18).

A. 74, G. pig 3875 (?), (in. 30/5/18; ill 25/6/18; d. 25/6/18).

A. 75, Sh. 3876 ( - ), d. in four days (in. 31/5/18; d. 4/6/18).

A. 76 (A. 63, A. 93, A. 115), Sh. 3877 ( - ), lumbar puncture, venous injection (in. 31/5/18).

A. 77 (A. 67, A. 89), Sh. 3878 ( - ), dried brain injected (in. 7/6/18)

*From* A. 72, M. 3873 (k. 15/6/18).

VIII. A. 78, M. 3890 ( + ), (in. 15/6/18; ill 25/6/18; k. 27/6/18)

A. 79, Kitten 3891 ( - ), (in. 15/6/18).

- A. 80, Sh. 3892 ( + ), (in. 17/6/18; ill 24/6/18; k. 28/6/18).  
 A. 81, Sh. 3893 ( + ). (in. 17/6/18; ill 29/6/18; k. 2/7/18).  
 A. 82, Sh. 3894 ( - , ? recovery), (in. 17/6/18; ill ? 29/6/18; d. 10/7/18)  
 A. 83 (A. 114), Sh. 3895 ( - ), (in. 17/6/18).  
 A. 84 (A. 109), Sh. 3896 ( - ), frontal and occipital only (in. 17/6/18).  
 A. 85 Sh. 3897 (death in 2 days). medulla only.

*From A. 75, Sh. 3876 (sheep histologically negative, d. 4/6/18).*

- A. 86 (A. 68, A. 90), Sh. 3879 ( - ), (in. 7/6/18).

*From A. 78, M. 3890 (k. 27/6/18).*

- IX.** A. 87, M. 3900 ( + ), (in. 27/6/18; ill 4/7/18; k. 7/7/18).  
 A. 88, Calf 3901 (recovered ?), (in. 27/6/18; ill 2-7/7/18).  
 A. 89 (A. 67, A. 77), Sh. 3902 ( + , recovering ?), (in. 27/6/18; ill 2-4/7/18; d. 4/8/18).  
 A. 90 (A. 68, A. 86), Sh. 3903 (recovered ?), (in. 27/6/18; ill 2 and 3/7/18)  
 A. 91 (A. 53), Sh. 3904 ( + ), (in. 27/6/18; ill 3/7/18; d. 3/7/18)  
 A. 92 (A. 70), Sh. 3905 ( + ), medulla only (in. 27/6/18; ill 3/7/18; d. 6/7/18).  
 A. 93 (A. 63, A. 76, A. 115), Sh. 3906 ( - ?), medulla only (in. 27/6/18; ill ? 2/7/18).  
 A. 94 (A. 69), Sh. 3907 ( - ), frontal and occipital only (in. 27/6/18).  
 A. 95, Horse 3908 ( + ), (in. 28/6/18; ill 7/7/18; k. 9/7/18).  
 A. 96, Sh. 3935 ( - ), (in. 29/7/18).

*From A. 81, Sh. 3893 (k. 2/7/18).*

- A. 97 (A. 16, A. 56), Dog 3909 ( - ), (in. 2/7/18).

*From A. 92, Sh. 3905 (d. 6/7/18)*

- X.** A. 98, Sh. 3910 ( + ), (in. 6/7/18; ill 11/7/18; d. 15/7/18).  
 A. 99, Sh. 3911 (death in 2 days).

*From A. 87, M. 3900 (k. 7/7/18).*

- A. 100, M. 3912 ( + ), (in. 7/7/18; ill 16/7/18; k. 17/7/18).  
 A. 101 (A. 125) Sh. 3913 ( - ), (in. 7/7/18).  
 A. 102, Sh. 3914 (recovered ?), (in. 7/7/18; ill 12-16/7/18).  
 A. 103, Sh. 3915 ( + ), emulsion plus serum of A. 82, Sh. 3894 (in. 7/7/18; ill 14/7/18; k. 17/7/18)  
 A. 104 (A. 126), Sh. 3916 ( - ), emulsion plus serum of A. 82, Sh. 3894 (in. 7/7/18).  
 A. 105, Sh. 3917 ( + ), emulsion plus normal sheep's serum (in. 7/7/18; ill 14/7/18; d. 16-17/7/18)  
 A. 106, Sh. 3918 ( - , death in 5 to 6 days), emulsion plus normal sheep's serum (in. 7/7/18; d. 12/7/18.)

*From A. 95, Horse 3908 (k. 9/7/18).*

- A. 107 (A. 127), Calf 3919 (recovered ?), (in. 10/7/18; ill on 13 and 14/7/18).

*From A. 98, Sh. 3910 (d. 15/7/18).*

- XI.** A. 108, Sh. 3920 ( + ), (in. 16/7/18; ill 22/7/18; d. 23/7/18)

*From A. 103, Sh. 3915 (k. 17/7/18).*

- A. 109 (A. 84), Sh. 3921 ( + ), (in. 18/7/18; ill 25/7/18; d. 27/7/18).

*From A. 100, M. 3912 (k. 17/7/18).*

- A. 110, M. 3925 ( + ), (in. 19/7/18; ill 30/7/18; k. 2/8/18, might have recovered)  
 A. 111, Sh. 3922 ( - ), emulsion plus serum of A. 89, Sh. 3902 (in. 19/7/18).  
 A. 112, Sh. 3925 (d. in four days), emulsion plus serum of A. 89, Sh. 3902.  
 A. 113, Sh. 3924 (d. in two days), emulsion plus serum of A. 89, Sh. 3902.

*From A. 109, Sh. 3921 (d. 27/7/18).*

- XII** A. 114 (A. 83), Sh. 3933 ( - ), (in. 29/7/18).

- A. 115 (A. 63, A. 76, A. 93), Sh. 3934 ( - ), (in. 29/7/18).

*From A. 110, M. 3925 (k. 2/8/18).*

- A. 116 (A. 132), M. 3936 ( - ), emulsion plus serum of A. 89, Sh. 3902 (in. 2/8/18).  
 A. 117, M. 3937 ( + ), emulsion plus serum of A. 102, Sh. 3914 (in. 2/8/18; ill 25/8/18; k. 27/8/18).



A. 118, Hen 3938 ( - ), (in. 2/8/18).

A. 119 (A. 130), Sh. 3939 ( - ), Berkefeld filtrate (in. 2/8/18).

A. 120 (A. 131), Sh. 3940 ( - ), Berkefeld filtrate (in. 2/8/18).

A. 121, Sh. 3941 ( + ), (in. 2/8/18; ill 8/8/18; d. 13/8/18).

A. 122, Sh. 3942 ( - ), (in. 2/8/18).

A. 123, Sh. 3943.

A. 124, M. 3952 ( + ), (in. 17/8/18; ill 3/9/18; k. 7/9/18).

*From* A. 121, Sh. 3941 (d. 13/8/18).

**XIII.** A. 125 (A. 101), Sh. 3948 (recovered ?), (in. 14/8/18; ill on 18 and 19/8/18).

A. 126 (A. 104), Sh. 3949 ( - ), (in. 14/8/18).

A. 127 (A. 107), Calf 3950 ( - ), (in. 14/8/18).

A. 128, M. 3951.

*From* A. 117, M. 3937 (k. 27/8/18).

A. 129, M. 3967 ( + ) (in. 27/8/18; ill 6/9/18, k. 9/9/18)

A. 130 (A. 115), Sh. 3968 (recovered?), (in. 28/8/18; ill 5-10/9/18).

A. 131 (A. 120), Sh. 3969 ( - ), (in. 28/8/18).

*From* A. 129, M. 3967 (k. 9/9/18).

**XIV.** A. 132 (A. 116), M. 3977 ( - ), (in. 11/9/18).

## ON THE EFFECTS OF INJECTIONS OF QUININE INTO THE TISSUES OF MAN AND ANIMALS.

BY LEONARD S. DUDGEON, C.M.G., C.B.E., F.R.C.P. (LOND.),

Colonel A.M.S., Consulting Bacteriologist B.S.F., and Member of the  
Advisory Committee to the War Office for Infectious Diseases  
and Sanitation, Eastern Mediterranean.

(With Plate III.)

IN December 1917, Lieut.-Col. MacGilchrist of the Indian Medical Service published a paper on the necrosis produced by intra-muscular injections of strong solutions of quinine salts<sup>1</sup>. It might be an advantage to quote the first few lines of this communication: "Advocates of intra-muscular injections of strong solutions of quinine salts for the treatment of malaria seldom omit to state that no local ill-effects are produced." He records a case of tissue necrosis following on the intra-muscular injection of eleven grains of quinine bi-hydrochloride in thirty-four minims of water. Death supervened thirteen hours later. MacGilchrist especially noted, owing to rapid tissue necrosis, that the track of the needle remained patent. He regards as an established fact that most of the quinine injected is precipitated and probably chemically combined with serum proteins in the necrosed tissues and for this reason intra-muscular injections of concentrated solutions of quinine salts are not to be recommended for cases of emergency. Very dilute solutions of quinine salts are, in his opinion, rapidly and completely absorbed whether employed subcutaneously or by the intra-muscular route. If the views which MacGilchrist puts forward in this and other communications are to be accepted without reserve then intra-muscular injections of strong solutions of quinine should no longer be employed. It was for this reason that Major-General Sir M. P. C. Holt, K.C.B., K.C.M.G., D.M.S., B.S.F., asked me to carry out an experimental enquiry on animals as to the effects produced by intra-muscular injections of strong solutions of quinine.

Human muscle was examined from fatal cases of malaria or suspected malaria which had received an injection of quinine at periods varying from one hour to three months from the time of the inoculation, and in some instances an estimation of quinine in the affected tissues was made. For the experimental enquiry, cast mules, rabbits, guinea-pigs, and frogs were used, while the preparations of quinine employed were (a) bi-hydrochloride in saline,

<sup>1</sup> *Indian Med. Gaz.* LII. No. 12.

(b) acid sulphate in saline<sup>1</sup>, (c) quinine alkaloid dissolved in alcohol (about the strength of hospital brandy), (d) and in ether (medicinal). The alkaloid shaken in blood serum and saline—a preparation which consisted of one gramme of alkaloid, one c.c. of 90 per cent. alcohol, and olive oil to 3 c.c.—and a somewhat similar preparation to the last mentioned was tested directly after it arrived in the East for trial. The quinine solutions have been injected in concentrated and dilute solutions. The preparations most commonly employed for intra-muscular injections were the bi-hydrochloride of quinine in saline, and, to a less extent, bi-hydrochloride dissolved in brandy. Human muscle was obtained in all instances from cases which had received quinine in some form in concentrated solutions as commonly employed for the treatment of malaria. Control observations were made on the action on the tissues of animals of acids and ether (quinine solvents).

Numerous cases were treated with intra-muscular injections of quinine bi-hydrochloride dissolved in brandy on the basis of three grains of the salt per 10 lb. of body weight.

Experiments have also been made on the absorption of quinine from the seat of inoculation, at periods varying from a few minutes to several weeks, and as to the question of the storage of the alkaloid in the heart-muscle, liver and kidneys.

All who have had experience of malarial patients are aware that an apparent anaemia occurs, and a true anaemia with considerable blood destruction. Further, a severe haemolytic anaemia and haemoglobinuria is associated with malaria and may occur at a period of the disease when quinine treatment is essential. For these reasons it was necessary to induce anaemia and haemoglobinuria in animals by means of immune sera so as to observe whether intra-muscular injections of quinine in concentrated and dilute solutions excited a more intense tissue reaction than in the control animals.

All the chemical estimations of residual quinine were undertaken by Captain C. E. C. Ferrey, O.B.E., R.A.M.C. (T.F.), Analytical Chemist to the Central Laboratory, B.S.F., who employed the Stas-Otto process for these investigations.

#### QUININE AND HAEMOLYSIS.

Although the purpose of these experiments was to observe the effects of intra-muscular injections of quinine on the tissues, yet, attention must be drawn to the haemolytic activity of quinine as estimated *in vitro*. Two solutions were prepared for the purpose: (1) 1 per cent. bi-hydrochloride of quinine in saline, (2) a solution of hydrochloric acid in saline of the same total acidity as the quinine solution—0.18 per cent. The total bulk of test solution and saline in each tube was 1 c.c. The haemolytic end point of the quinine solution, acting at 37° C. for five hours, was 0.16 c.c., while the acid solution alone induced haemolysis down to 0.1 c.c. Normal saline was employed throughout

<sup>1</sup> Only three experiments were made with this salt as it was found to excite more intense necrosis than the other preparations which were employed.

the experiments as the diluting agent. Further, the haemolytic action was much more rapid with the acid solution than with the bi-hydrochloride of quinine in saline. Normal human serum or citrated plasma in suitable amounts prevented haemolysis. These results briefly referred to serve to illustrate the haemolytic action of bi-hydrochloride of quinine and of the corresponding acid. It will be readily appreciated that the preparations of quinine employed for intra-muscular injections in the treatment of malaria are of considerably greater strength than in these experiments on haemolysis *in vitro*. The haemolytic activity of a 1 per cent. solution of bi-hydrochloride of quinine is extremely rapid, while strong solutions produce instantaneous haemolysis. The alkaloid when suspended in saline induces a much more gradual haemolysis than the salt in solution, although for obvious reasons a considerable error must occur in estimating the haemolytic action of varying suspensions of quinine alkaloid in saline. It has been found that the agglutination of human or other red cells, well known to be induced by free acids, is absent in the standard acid solutions of bi-hydrochloride of quinine employed in suitable strengths for such purposes. If 0.05 c.c. of the standard solution of hydrochloric acid already referred to is made up to a total volume of 1 c.c. an immediate agglutination occurs on the addition of human red cells, while with 0.15 c.c. of the 1 per cent. solution of bi-hydrochloride of quinine in 1 c.c. saline no such effect is induced. All experiments tend to show that although quinine acts as a powerful haemolytic agent the acid employed to dissolve the alkaloid is much more potent in its effect.

#### WHAT IS THE COMPARATIVE EFFECT OF INTRA-MUSCULAR INJECTIONS OF CONCENTRATED AND DILUTE SOLUTIONS OF QUININE?

Numerous experiments have been made to solve this question concerning which information was specially required.

The following detailed descriptions express the essential features:

(1) A rabbit received an intra-muscular injection of 0.039 grammes of bi-hydrochloride of quinine in 1 c.c. saline, and the same quantity of quinine in 3 c.c. of saline into another set of muscles.

The following day 0.078 grammes of the same preparation in 2 c.c. of saline was injected in the right side and a similar quantity in 4 c.c. of saline in the left.

The post-mortem examination took place on the following day. The results showed that no advantage was gained by injecting the quinine in twice the quantity of saline. The spreading oedema was greater owing to the increase in bulk of fluid injected, while the inflammatory process and muscle necrosis in both instances was so evident that no importance could be attached to minute differences in the affected tissues. If quinine injections are given in such dilutions that the action of quinine and free acids on the red cells and tissues is reduced to a minimum, then the bulk of fluid required would nullify, in my opinion, any advantage that might be gained. Further there is a greater

possibility of suppuration from injection of quinine in large bulk into the tissues at frequent intervals.

(2) Four rabbits were injected by the intra-muscular route as follows:

- A. 0.018 gramme of bi-hydrochloride of quinine in 0.5 c.c. saline (concentrated).
- B. 0.018 gramme of bi-hydrochloride of quinine in 0.5 c.c. normal rabbit serum (concentrated).
- C. 0.018 gramme of bi-hydrochloride of quinine made up to 2 c.c. with auto-rabbit serum.
- D. 0.7 c.c. of 1:5000 solution of quinine alkaloid in ether.
- E. 0.18 gramme of bi-hydrochloride of quinine made up to 2 c.c. with auto-rabbit serum and injected by the intra-muscular route.

In every instance obvious necrosis occurred. The interval between the time of the injection and the autopsy was six days in the case of experiments A and B, 4 days in C and E, and 3 days in D.

The most intense changes were induced when the alkaloid was injected with ether. Necrosis of muscles in all stages—wide areas of polynuclear and mononuclear inflammation especially towards the capsular area—thrombosis of vessels—red cell agglutination and haemolytic changes in the tissue blood as well as in the intra-vascular blood.

(3) A mule was injected with 1 gramme of alkaloid of quinine in alcohol and olive oil (3 c.c.). On the following day with 1 gramme of the alkaloid in 10 c.c. ether.

Although the total bulk of fluid varied considerably in each inoculation the final results were similar; further, the concentration of quinine in the solutions employed did not affect the final results. In each instance extensive necrosis—haemorrhage into the tissues—congestion of blood vessels—and foci of acute inflammation were met with. Numerous experiments have been referred to elsewhere in this communication which serve to illustrate the comparative effects of concentrated and dilute solutions when injected into the tissues of animals.

#### THE MICROSCOPICAL FINDINGS AS A RESULT OF INTRA-MUSCULAR INJECTIONS OF QUININE SALTS AND ALKALOID QUININE.

The first effect of an injection of a quinine preparation into the tissues is necrosis of muscle. The fibres most affected are completely necrosed leaving the empty sheaths. Oedema of the tissues accompanies the necrosis, together with agglutination of red cells and haemolysis in the vessels and in the blood which has escaped into the tissues. These changes are in evidence within ten minutes of the inoculation. At this period no leucocytic reaction has occurred, but an intense congestion of all vessels in the area has taken place. At the end of one hour the leucocytic reaction may not be a marked feature, although in preparations of quinine which include oil an intense polynuclear leucocytic reaction has been observed. The muscle fibres in the necrotic areas, as time advances, present appearances which are various as well as distinctive. In addition to the fibres which are completely necrosed others show fragmentation so that numerous "apparent droplets" are observed within the sheaths (Pl. III,

Fig. 1). Owing to a multiplication of the nuclei of the sheaths which occurs and a collapse of the sheaths themselves, the fibrous framework of the new tissue is gradually formed. Shrunk fibres with several nuclei give the well-known appearance of pseudo-giant cell formation. The large tissue cells act as phagocytes for fat droplets, red cells and free iron granules. Active destruction of the vessel walls occurs together with thrombosis which may or may not serve as a conservative process (Pl. III, Fig. 3). Polynuclear inflammation of the necrosed walls can be clearly demonstrated. Active changes occur in the vessels apart from thrombosis such as agglutination of red cells and haemolytic phenomena while similar changes are met with outside the vessel walls. In certain instances marked red cell agglutination occurs within the vessel without the evidence of inflammation (Pl. III, Fig. 2).

In course of time absorption of muscle fragments and of inflammatory products takes place so that finally we have a condition of fibrous myositis such as is met with as a result of traumatic influence or syphilis. Nerve fibres are implicated in the earliest stages. It is not uncommon to observe that haemorrhages and large vacuoles occupy the space of the original nerve fibres. Fibrous tissue formation surrounds the nerves in the muscles and also the individual fibres. Large nerve trunks may be so implicated by the oedema and spreading necrosis that complete nerve degeneration occurs.

Strands of necrosed muscle fibres may persist for ten to fourteen days after an injection of quinine, possibly owing to the diminished tissue absorption which must occur as a result of quinine inoculation. The bulk of quinine however is absorbed from the tissues with extreme rapidity—simply a question of hours.

#### EXPERIMENTS TO ILLUSTRATE THE EFFECTS ON THE TISSUES OF CERTAIN PREPARATIONS OF QUININE.

A cast mule was injected with the following preparations. The results were as follows:

Number of inoculation	Site of inoculation	Nature of inoculation	Naked eye appearances at autopsy	Interval between inoculation and examination
1	Back, near side	10 c.c. ether	Extensive necrosis, 6 or 7 inches long, 3-4 inches wide. Slight superficial oedema	6 days
2	Near fore leg	ditto	Extensive tissue necrosis. Slight oedema	5 days
3	Back, off side	0.25 grm. of alkaloidal quinine in 10 c.c. ether	Widespread oedema. Very extensive necrosis	4 days
4	Off fore leg	ditto	ditto	3 days
5	Near hind leg	5 c.c. of 4.3 % HCl in saline	Extensive oedema spreading along muscle septa. Marked necrosis	2 days
6	Off hind leg	5 c.c. of 25% bi-hydrochloride of quinine in saline (same total acidity as above (5))	ditto	24 hours

This experiment gives an excellent survey of the effect produced by ether-quinine alkaloid dissolved in ether—bi-hydrochloride of quinine in saline—hydrochloric acid made to the same total acidity as the solution of quinine. In Experiment 3 of this series 0.25 gramme of alkaloidal quinine was injected in no less than 10 c.c. ether, yet the effect on the tissues was considerable. Here the quinine was given in dilute solution, but the solvent employed was largely responsible for the effect. Experiments 5 and 6 of this series show similar effects on the tissues, yet quinine was not employed in Series 5. The result must have been due therefore to the solvent. This fact must never be lost sight of in any discussion on the question of tissue necrosis induced by quinine, as will be referred to elsewhere in this communication.

#### INTRA-MUSCULAR INJECTIONS OF ALKALOIDAL QUININE IN 60 PER CENT. ALCOHOL.

Several animals received intra-muscular injections of quinine dissolved in alcohol, either concentrated or in dilute solutions. The rabbit used in the experiment to be referred to in detail was injected daily for six days with 0.04 gramme in 1 c.c. of 60 per cent. alcohol. Blood counts were made with the object of determining whether a leucocytosis would be induced or an alteration in the red cell-haemoglobin system. The injections were made in a different muscular area on each occasion. Certain tissues were preserved at the autopsy for quinine estimation, and the entire local area was removed for a similar purpose except for a small portion reserved for microscopy. First injection: there was marked oedema and a long line of muscle necrosis. A typical fibromyositis had been produced around the necrosed muscle in which all possible changes were recognised. Numerous new formed blood vessels were present, also blood pigment and scattered haemorrhages. Degeneration of the vessel walls was evident. The effect of the second inoculation was similar except that a well-developed interstitial neuritis was found in the nerve from the affected area. No quinine was detected in the local lesion. The third injection had produced a large haemorrhagic area superficial to a diffuse black-brown necrosis of muscle. Numerous foci of polynuclear cells were present. No quinine was obtained from the local lesion. Fourth injection made four days before death showed very considerable oedema of muscle and necrosis. Thromboses of some of the large vessels were present with necrosis of the muscular walls. The degenerated muscle fibres were fragmented and were undergoing a process of gradual absorption by which highly cellular patent sheaths remained, the walls of which ultimately coalesced and thus assisted in the formation of the fibrous scar.

Fifth injection. Diffuse haemorrhage and oedema together with a prominent area of greyish brown, dry, muscular necrosis had occurred.

Sixth injection. This was completed forty-eight hours before death. The changes noted at the autopsy were similar to those referred to as a result

of the previous inoculation. The microscopical appearances were similar, but of a more acute type. There was a very marked polynuclear inflammation, abundant haemorrhages, and active hæmolytic changes. The muscle fibres were necrosed, fragmented, and broken up into coarse granules, and haemorrhages had occurred among the nerve bundles. Both kidneys and liver removed at the autopsy forty-eight hours after the last intra-muscular injection were free from quinine, although 0.24 gramme of alkaloidal quinine had been injected in eight days, and the last injection was only forty-eight hours before death. It will be readily appreciated from those experiments described in detail that alkaloidal quinine given in 60 per cent. alcohol excites intense changes in the tissues in the inoculated area.

*Intra-muscular Injections of Quinine Alkaloid in Alcohol*

Dates	Weights	Red cells per c mm	H B	C I (Colour- Index)	Leuco cytes per c mm	Differential count of leucocytes 1st column = %, 2nd column no per c mm								Quinine alkaloid in 60 % alcohol Intra-muscular injections. Total bulk of fluid = 1 c c
						Nucleated red cells No. to %	Finely granular oxophil	Lympho- cytes	Large hyalines					
1	2	1	2	1	2									
15/7/18	1000 grms.	5,400,000	83 %	0.7	8980	2	43.0	3827	55.0	4895	1.5	133	0.04	gramme
16/7/18	950 „	—	—	—	7900	1	48.5	3831	50.0	3950	1.0	79	0.04	„
17/7/18	950 „	5,200,000	84 %	0.8	7600	3	27.5	2190	38.0	5168	3.5	266	0.04	„
18/7/18	955 „	—	—	—	7020	1	32.5	2275	61.0	4270	5.5	385	0.04	„
19/7/18	1000 „	5,200,000	82 %	0.7	7100	3	27.5	1952	65.0	4615	6.5	461	0.04	„
20/7/18	1000 „	5,300,000	82 %	0.7	7150	—	—	—	—	—	—	—	0.04	„
21/7/18	950 „	5,000,000	80 %	0.8	6990	1	27.0	1863	63.0	4347	10.0	690		
22/7/18	923 „	5,200,000	84 %	0.8	7520	0	22.0	1650	68.0	5100	8.0	600		Rabbit killed

THE INJECTION OF QUININE ALKALOID WITH OLIVE OIL.

The following preparation was tried for intra-muscular injections:

Quinine alkaloid   ...   ... 1 gramme.  
 Alcohol 90 per cent.   ...   ... 1 c.c.  
 Olive oil (neutral)   ...   ... to 3 c.c.

Injections were made of 0.2 c.c. of the above mixture into rabbits. Some animals received one injection, others as many as five on successive days. The chief features in such experiments are concentration of the alkaloid and the introduction of a fatty substance on the assumption that it would act as a tissue protector. To avoid needless repetition it will be sufficient to refer to one experiment in detail.

A rabbit received five intra-muscular injections of the alkaloid in oil commencing with 1/10 c.c. containing half a grain of quinine on five successive days. Each inoculation was made into a different area of fresh tissue. The animal was killed eleven days from the first commencement of the experiment. During the period it lost 160 grammes in weight. No advantage would be gained by recording the tissue changes met with in each muscular area. The



naked eye appearances were profound in each focus as occurs with all concentrated injections of quinine, while the oil exerted no beneficial action. On the other hand an injection of one grain of the alkaloid for a rabbit must be regarded as a full dose. The muscles were soft, showed large areas of necrosis, thrombosis of vessels and haemorrhages into the muscular tissue. The red haemorrhagic zone was sharply defined from the necrosed pale area.

Microscopically an extensive fibro-myositis had developed with diffuse muscle necrosis. A polynuclear inflammation was far more in evidence than has occurred in experiments with other preparations of quinine. Nerve fibres embedded in the muscle tissue were degenerated, while in the older lesions fibrous tissue could be recognised among the fibres and surrounding them.

The second injection amounted to 1 grain of the alkaloid in a total bulk of 1/5 c.c. It had the advantage, therefore, for this experiment of concentration. The inoculation was made in the muscles in the front of the thigh. At the autopsy 10 days later it was found that the necrosis had spread to the muscles at the back of the thigh and the great sciatic nerve was surrounded by necrosed and inflammatory tissue. The nerve was removed entire and sections were prepared in "Marchi" at various levels. An extreme degree of nerve degeneration was noted, in fact, very few of the fibres escaped (Pl. III, Fig. 4). Some were completely degenerated, others patchy, while actual haemorrhages into the nerve had taken place. Here we have an example of the destruction of the sciatic nerve following an injection of quinine. The inoculation was made distant from the nerve, directly into a muscle, and in such a manner that escape of fluid was well-nigh impossible. The necrosis, however, was so excessive that it had extended right through the thigh muscles and implicated the sciatic nerve with disastrous results for the animal. This fact, however, will be referred to later. The entire liver and both kidneys were examined for evidence of quinine storage, but no alkaloid was detected.

#### THE INJECTION OF QUININE ALKALOID IN CREOSOTE AND FAT.

A preparation which was sent to Macedonia for trial consisting of one gramme of quinine alkaloid, 0.75 c.c. of beechwood creosote and 5 c.c. of neutral fat was not found to possess any special advantage over the other preparations referred to. Mules received intra-muscular injections of the above preparation causing extensive necrosis, and a gelatinous spreading subcutaneous oedema. A lesion some  $6\frac{1}{2}$  inches long, 3 inches deep, and  $2\frac{1}{2}$  inches wide was produced by the injection of 10 c.c. into the muscles of a mule. Chemical analyses of the necrosed muscle showed that the quinine had been absorbed with rapidity. Two experiments will be quoted to illustrate this point.

	Amount injected	Date	Post-mortem date	Amount of quinine recovered
Experiment 1.	5 c.c.	21st	25th	0.003 gramme
"      2.	5 c.c.	22nd	25th	0.063    ,,

Similar evidence in the case of human muscle was also obtained.

*A cast brown horse was inoculated intra-muscularly with bi-hydrochloride of quinine in saline as illustrated in the accompanying table :*

No. of experiment	Amount of bi-hydrochloride of quinine employed	Interval between inoculation and autopsy	Naked eye appearance of the tissues at the autopsy
1	2.5 grms. in 5 c.c. saline	13 days	Marked necrosis and fibrosis.
2	1.125 grms. in 2.5 c.c. saline	8 days	Localised necrosis separated from a fibrous zone by a line of haemorrhage
3	4.5 grms. in 10 c.c. saline	3 days	Long tract of necrosis with marked oedema of muscle
4	2.25 grms. in 5 c.c. saline	2 days	Localised necrosis with oedema
5	1.125 grms. in 2.5 c.c. saline	30 hours	Very extensive gelatinous oedema with localised necrosis
6	1.125 grms. in 2.5 c.c. saline	1½ hours	Localised necrosis with wide tracts of haemorrhage

A brief account of the microscopical appearances of the lesions referred to in the previous table will be recorded.

No. 1. Fibrous myositis with complete capsulation of the necrotic muscular tissues. Active polynuclear inflammation present.

No. 2. Fibrous myositis completely enclosing necrosed muscle—haemorrhagic foci and polynuclear inflammation.

No. 3. Extensive necrosis of muscular tissue and of the walls of blood vessels resulting in haemorrhage and active haemolytic changes in the diffused blood. Agglutination of red cells very obvious.

No. 5. Wide necrosis of muscular tissue and an intense leucocytic reaction in and around muscle fibres which are fissured, fragmented, and show all stages of degeneration. Active haemolytic changes and masses of agglutinated red cells present.

No. 6. Diffuse muscle necrosis with long tracts of oedematous tissue evident. Haemorrhagic foci and active haemolytic phenomena present. Walls of blood vessels necrosed and ruptured.

*A cast grey mule was injected intra-muscularly with certain solutions of quinine with the following results :*

No. of experiment	Amount of quinine injected and total bulk of fluid	Amount obtained at autopsy	Interval between the intra-muscular injections and the post mortem examination	Appearances of the muscle lesions at the autopsy
1	4.1 grms. alkaloid in 10 c.c. of 70 % alcohol	0.014 gramme	8 days	Fibrosis and extensive necrosis
2	4.5 grms. bi-hydrochloride in 10 c.c. saline	0.020 "	7 "	Central black necrosis, scattered haemorrhages. Gelatinous oedema
3	2.25 grms. bi-hydrochloride in 5 c.c. saline	0.003 "	6 "	Central necrosis with haemorrhagic zone at periphery. Gelatinous oedema
4	Ditto	0.0085* "	5 "	Ditto (Pl. III, Fig. 5)
5	1.125 grms. bi-hydrochloride in 2.5 c.c. saline	0.012 "	4 "	Changes similar, but lesions less extensive
6	4.1 grms. alkaloid in 10 c.c. of 70 % alcohol	0.074 "	3 "	Extensive gelatinous oedema surrounding a wide area of dry necrosis (7)
7	2.05 grms. alkaloid in 5 c.c. of 70 % alcohol	0.048 "	2 "	Central black necrosis with oedematous tissue around
8	2.25 grms. bi-hydrochloride in 5 c.c. saline	0.938 "	1 hour	Extensive dry necrosis about 4 inches square

\* This does not represent the sum total because some of the extract was blown out of a flask.

In each instance a different area of the animal's body was used for the injection.

The mule was killed one hour after the last inoculation. At the post-mortem examination, the affected tissues in the region of the injections and some of the surrounding healthy tissue were removed for estimation of quinine content, while a small portion was reserved for microscopy.

We can infer from the results of the chemical investigation of the muscle lesions as illustrated in the accompanying table that the bulk of the quinine is absorbed whether injected as an alkaloid in alcohol, or as the bi-hydrochloride in saline. It was also found that at the expiration of one hour from the time of the inoculation to the death of the animal only 0.938 gramme of alkaloid was recovered out of 2.25 grammes injected.

It may be an advantage while referring to the rate of quinine absorption to cite the following experiment.

0.1 gramme of bi-hydrochloride of quinine in 0.25 c.c. saline was injected into the thigh muscles of both hind legs of a guinea-pig; ten minutes later a similar quantity was inoculated into the muscles of the left fore leg, and the animal was killed immediately. The left hind leg was amputated at the hip joint and handed to Captain Ferrey entire. He obtained 0.061 gramme of alkaloid from the tissues which amounts to 0.075 gramme of the bi-hydrochloride. The tissues in the injected areas showed spreading oedema and marked necrosis of muscle. Agglutination of red cells and haemolytic changes had occurred. No tissue reaction was present.

The tissues examined *immediately* after the intra-muscular injection were oedematous and the muscles showed evidence of necrosis.

#### MICROSCOPICAL CHANGES IN THE AFFECTED TISSUES.

*Lesions Nos. 1 and 2.* The tissue changes in the affected muscles which are of eight and seven days standing respectively were similar although quinine alkaloid was injected in the first case and quinine bi-hydrochloride in the second. Microscopically there was typical fibrous myositis with diffuse muscle degeneration. The abundance of nuclei in the muscles among the fragmented fibres was very apparent, and in some instances gave the well-known appearances of pseudo-giant cell formation. Large haemorrhages were observed more especially in relation to strands of necrosis. Mononuclear cells were numerous throughout the new-formed fibrous tissue, and foci of polynuclear phagocytes. The free iron reaction was demonstrated, and spider cells lying in the fibrous tissue were filled with these granules.

*Lesions Nos. 3 and 4* resulted from the injection of quinine bi-hydrochloride on successive days. The fibrous tissue formation was somewhat similar to that which has been referred to in the case of the first two experiments. Large tracts of structureless walls filled with tinged serum were abundant, as also scattered haemorrhages and phagocytosis of red cells by large mononuclear cells. Necrosis of muscle and splitting up of muscle fibres were more evident than in the first two experiments.

*Lesion No. 5.* Extensive necrosis of muscle and "vacuolation" had occurred. Some of the larger blood vessels were thrombosed and complete necrosis of the muscular walls was evident while other vessels showed intense polynuclear inflammation. Strands of polynuclear cells were scattered all through the connective tissue stroma.

*Lesion No. 6.* The most striking feature in this experiment was a zone of necrosed muscle with fibrosis divided from a layer of vacuolated and distorted muscle cells by a broad line of haemorrhage, while acute oedema extended for some distance between the fibres.

*Lesion No. 7.* This lesion showed two distinct pathological changes:

(1) Haemorrhagic area which contained tracts of free blood and congestion of blood vessels together with areas of haemolysed blood in which were present agglutinated red cells. Intense polynuclear inflammation had occurred in the vessel walls and all stages of muscle degeneration.

(2) Necrotic area. The contrast with the vascular area was most marked. Extensive necrosis of muscle with outlying fibres swollen, vacuolated, and distorted and necrosis of the muscular walls of the large blood vessels was found. Throughout the whole tissue there was polynuclear inflammation.

*Lesion No. 8.* This was produced at the end of one hour by one injection of 2.25 grammes of bi-hydrochloride of quinine. Wide tracts of haemorrhage occurred along the muscle bundles with acute oedema spreading in all directions. Muscle fibres were necrosed and in all stages of degeneration. In one portion of the affected muscles wide areas of haemorrhage had occurred with direct destruction of large blood vessels. In this area intense polynuclear inflammation was present while in an adjoining area leucocytic reaction was absent. Rings of polynuclear cells were in evidence in the sheath of the muscle fibres, infiltrating the fibres, and dividing them up into large granular masses. Wide areas of lysed and partly lysed red cells were present. Phagocytosis of red blood corpuscles by large mononuclear cells was very evident, also clumps of agglutinated erythrocytes.

#### FIXATION OF QUININE IN THE TISSUES.

These experiments were undertaken at the suggestion of Captain J. F. Gaskell, R.A.M.C., so as to ascertain whether quinine when injected into the muscles becomes immediately fixed locally, as if so, quinine "absorption" from the tissues would be only an apparent effect.

*Experiment 1. Cast Mule.* 1.0 gramme of alkaloid quinine was injected as the bi-hydrochloride into the belly of a leg muscle which had been exposed for this purpose. The blood vessels were tied immediately by Captain Moir, A.V.C., and the whole muscle and tendons were removed without delay. The chemical analyses were made by Captain F. S. Hele, M.D., R.A.M.C., and the results were as follows:

Watery extract, 0.217 gramme.

In muscle, 0.595 gramme.

*Effects of Quinine*

In cloth (used in the experiment), 0.026 gramme.

Total quantity of quinine alkaloid obtained from the muscle, 0.838 grm.

Experiment 2. Similar experiment to No. 1 except that the muscle remained in the body for twenty minutes after the blood vessels were tied by Captain Moir, A.V.C.

Chemical analysis by Captain Hele, R.A.M.C.

Watery extract, 0.307 gramme.

In muscle, 0.460 gramme.

In cloth, 0.029 gramme.

Total quantity of quinine alkaloid extracted from muscle, 0.796 gramme.

The results of these experiments do not suggest that quinine is fixed in the tissues immediately after inoculation to any appreciable extent. Captain Hele considers that the amount "lost" in the above experiments was due to technical difficulties.

THE EFFECTS OF INTRA-MUSCULAR INJECTIONS OF QUININE IN ANIMALS  
RENDERED ANAEMIC.

These experiments were undertaken to ascertain whether intra-muscular injections of quinine produced more severe tissue changes in animals in which a severe haemolytic anaemia had been induced than in control animals. The anaemia and haemoglobinuria were induced by injecting rabbits with the serum of a cat which had been immunised with rabbit's cells for the purpose of these experiments. The immune serum was injected intravenously. Blood counts were made at daily intervals and the weights of the animals were carefully recorded. It was necessary to have records of these data because many patients who receive intra-muscular injections of quinine are anaemic and some are suffering from various grades of haemolytic toxæmia. The most exhaustive experiment will be referred to in detail. Intra-muscular injections of bi-hydrochloride of quinine in saline were given in varying amounts from large to excessive doses with the object of exciting more severe tissue changes in the anaemic animals than in the control. The quinine injections were made previous to and during the period of severe anaemia. There was no leucocytosis. On the contrary a definite reduction in the total white cells occurred, followed by a rise to the total previous to the inoculation as the condition of the blood improved. A full record of the three most important varieties of white cells are given, but the only noteworthy feature is the absolute increase in larger hyaline cells, as met with in malarial fever. The animal showed the effects of each injection, but no more so than normal rabbits which received similar inoculations. When the animal was killed one month from the commencement of the experiment the sites of the quinine inoculations were represented as patches of scar tissue which were most obvious in the case of the excessive dose of 0.6 gramme given thirty days before the death of the animal. The results of the microscopical examination of the scar tissue were similar in

each instance. Fibro-myositis—necrosed muscular tissue—scattered round celled inflammation, well shown as a perivascular effect—and small foci of polynuclear inflammation were the conspicuous features of the various lesions. To quote this experiment is sufficient for the purpose intended, as it is definitely shown that quinine when given by the intra-muscular route in con-

Dates	Weights	Red cells per c.mm.	H B.	C I (Colour- Index)	Leuco- cytes per c.mm.	Differential count of leucocytes.						Doses of quinine bi-hydrochloride in saline Total bulk in- jected on each occasion = 1 c.c.
						Nucleated Red cells No. % of leucocytes	1	2	1	2	Large lymphocytes	
29/5/18	1510 grms.	6,000,000	100 %	0.8	7500	0	21.9	1642	76.0	5700	0	0
30/5/18	1490 "	—	—	—	—	—	—	—	—	—	—	—
31/5/18	1490 "	—	—	—	—	—	—	—	—	—	—	—
1/6/18	1510 "	6,000,000	100 %	0.8	6400	0	32.6	2086	66.0	4224	0.6	0.25 grammes
2/6/18	1515 "	—	58 %	—	—	0	13.0	—	85.3	—	—	0.45 "
3/6/18	1445 "	4,300,000	50 %	0.5	7200	6.0	20.0	1440	79.3	5709	0	0
4/6/18	1400 "	1,500,000	32 %	1.0	5000	7.0	27.0	1350	72.3	3615	0.6	0.09 "
5/6/18	1400 "	1,500,000	32 %	1.0	4500	2.0	34.0	1530	65.3	2938	0	0
6/6/18	1390 "	2,000,000	36 %	0.9	5200	1.0	55.0	2860	44.6	2319	0	0.045 "
7/6/18	1300 "	2,056,250	40 %	1.0	5400	4.0	34.3	1812	56.6	3056	4.3	232
8/6/18	1330 "	2,170,000	35 %	0.8	5000	16.5	60.0	3000	40.0	2000	0	0
9/6/18	1335 "	3,280,000	65 %	1.0	4900	10.0	30.0	1470	69.0	3381	1.0	49
10/6/18	1365 "	3,290,000	65 %	0.9	6800	2.5	32.0	2176	67.5	4590	0.5	34
11/6/18	1320 "	4,200,000	66 %	0.7	7260	2.0	42.0	3024	55.0	3960	2.0	144
12/6/18	1350 "	4,290,000	65 %	0.7	7280	2.0	29.0	1988	66.0	4752	5.0	360
30/6/18	1610 "	4,496,875	82 %	0.9	7250	0	31.0	1532	58.0	4176	9.0	648

centrated and massive doses excites no greater reaction in the tissue of animals rendered anaemic than in the case of the normal, while no general effect occurred even when the inoculations were made on successive days. Other experiments completed on these lines led to similar conclusions. One rabbit,

in which a severe experimental anaemia had been induced, received two massive intra-muscular doses of quinine during the period of haemoglobinuria, but the effects were similar to those obtained in the control animals, while a diminution in the total leucocytes was recorded.

#### HUMAN MUSCLE.

Muscle tissue from the region of a previous quinine inoculation has been examined from several cases in this Command. The interval between the inoculation and the examination varied from so short a period as one hour up to three months. It has been suggested that the effects of quinine on the tissues is not capable of demonstration until twenty-four hours from the time of the inoculation. This view is erroneous on experimental evidence while it is equally fallacious in the case of a human subject. A man, comatose from malarial fever, was admitted to hospital, an intra-muscular injection of twenty grains of bi-hydrochloride of quinine was given, but the patient died one hour later. At the autopsy a large area of black green necrosis about four by four inches surrounded by gelatinous oedema was discovered at the seat of inoculation. All cases which I have had the opportunity to examine have received concentrated quinine. Experimentally, quinine injections can be given and the autopsy performed immediately, but the neurotic action is quite obvious. No detailed description of the microscopical changes will be given in this section except such as refer to points of special importance. Certain cases of special interest will be briefly referred to so as to illustrate the most essential features as regards quinine inoculation. Twelve grains of bi-hydrochloride of quinine were injected into the right buttock forty-eight hours before death. At the autopsy a large area of complete necrosis of muscle was observed together with a wide tract of haemorrhage due, as was proved on microscopical examination, to complete destruction of the wall of a large artery. The entire necrosed muscular tissue together with that tissue in immediate contact was examined chemically, except for a small portion reserved for microscopy, but no quinine was detected. Two intra-muscular injections of fifteen and twenty grains respectively had been made at an interval of twenty-four hours and death occurred about twenty hours later. The resulting lesion was similar in each case—large area of necrosis, a band of haemorrhage and congestion, with a wide tract of gelatinous oedema.

In the case of a Greek labourer who had received an intra-muscular injection of fifteen grains of bi-hydrochloride of quinine twenty-two hours before death, only 0.02 gramme of the alkaloid was obtained from a large area of necrosis at the seat of the inoculation. The absorption here was rapid in spite of the fact that the patient was dying from fulminating pneumococcal septicaemia.

Certain cases of malarial fever with malarial parasites present in the circulating blood were complicated with blackwater fever, but the effects from intra-muscular injections of bi-hydrochloride of quinine were similar to those

observed in the uncomplicated cases, except that the haemorrhagic zone appeared darker in colour, almost black in some instances (Pl. III, Fig. 6).

One of the most important cases which illustrate the action of quinine occurred in the case of a man who died from blackwater fever and malaria. Four days before death he received an intra-muscular injection of twenty grains of bi-hydrochloride of quinine. The inoculation was made into the same buttock which had been injected three months previously with a similar dose for malarial fever. This man had complained of aching and cramping pains in the buttock at the site of the inoculation, especially if he sat for any length of time on a hard seat or had prolonged exercise. At the autopsy intense necrosis had occurred at the site of the recent injection which merged into the old lesion, which showed dense fibrous tissue at the centre of the focus, and a definite broad band of fibrous myositis at the periphery. Acute polynuclear inflammation from the recent injection had extended to this layer of fibrous myositis. The pain referred to could be explained by the patch of dense fibrous tissue in the centre of an important muscle surrounded by a zone of fibrous myositis, but further, owing to a fibro-neuritis which existed in this area. Some of the nerves of the muscle were surrounded by dense fibrous tissue, and similar foreign tissue had replaced many of the nerve fibres. Similar examples were met with in cases of much shorter duration, and also experimentally, a fibro-myositis, and fibro-neuritis.

A patient was admitted to hospital with malignant malaria. He received an intra-muscular injection of 1.33 grammes of bi-hydrochloride of quinine but died two hours later. The whole of the necrotic tissue, which was three inches square, surrounded by oedema, was removed for chemical investigation except for a small portion reserved for microscopy. There was extensive necrosis of muscle, necrosis of the walls of blood vessels, marked evidence of haemolysis, but no acute inflammation was present.

Although the patient was inoculated when *in extremis*, only 0.344 gramme of alkaloidal quinine was extracted from the tissues.

The fact that necrosis of the tissues always accompanies the intra-muscular or subcutaneous injections of quinine is not realised sufficiently by Medical Officers, even those who have employed these methods on a large scale. No better illustration of the correctness of this statement can be furnished, than by quoting the following instance. Owing to certain bad results which had occurred from intra-muscular injections of quinine, an Army order was issued to the effect that all Divisional Officers must report in detail to the D.M.S. any ill effects subsequent to intra-muscular injections of quinine. One Divisional Officer after fifteen months' experience of this method of treatment of malaria furnished a report to the D.M.S. to the effect that a man had died from malaria and at the post-mortem examination a wide area of necrosis of muscle was found at the seat of injection. He concluded his evidence with the statement that all Medical Officers in his unit had been warned of this unfortunate incident and that every effort would be made to prevent a



recurrence of this disaster! I have discussed the question of quinine necrosis with innumerable Medical Officers who have had wide experience of intra-muscular injections of quinine and it is by no means uncommon to learn from them that they were unaware that such effects occurred in the tissues apart from negligence. It is this lack of knowledge of the methods of quinine administration which serves to explain the cause of many of the disasters which have occurred. It is, therefore, necessary to emphasize that quinine injections should not be given in the vicinity of large nerve trunks, or main arteries, that the injections should not be repeated in the same area of muscular tissue and that this method of quinine administration should only be employed when circumstances demand it<sup>1</sup>.

The chief complications of intra-muscular injections of quinine in the human subject during 1916-17 and '18 of which I have records were as follows: (1) Tetanus, (2) Gangrene, (3) Abscess formation, (4) Pyaemia, (5) Nerve Palsies, (6) Haemorrhage from large Arteries, (7) Sciatica, (8) Chronic muscular pain, (9) Pain and deficient movements in affected muscles, (10) Thrombosis in varicose veins.

#### THE RESULTS OF THE ESTIMATION OF QUININE ALKALOID IN HUMAN MUSCLE SUBSEQUENT TO QUININE INJECTIONS.

No. 1, Broncho-pneumonia. Intra-muscular injections of 21 grains of bi-hydrochloride of quinine into left buttock. Two days later 26 grains were injected on the opposite side. Death on the following day. Amount of quinine alkaloid recovered from first injection, 0.227 gramme, and from the second injection, 0.345 gramme.

No. 2, Malignant malaria. Intra-muscular injection of 15 grains of bi-hydrochloride of quinine. Death four days later. Amount of quinine alkaloid recovered, 0.002 gramme. This patient had received ten intra-muscular injections in fourteen days amounting to 115 grains, and six intra-venous injections which totalled 55 grains.

No. 3, Malignant malaria. Intra-muscular injection of 10 grains of bi-hydrochloride of quinine. Death three days later. Amount of quinine alkaloid recovered from muscle, 0.0115 gramme.

No. 4, Lobar pneumonia. Intra-muscular injection of 15 grains of bi-hydrochloride of quinine. Death 24 hours later. Amount of quinine alkaloid recovered from muscle, 0.048 gramme.

No. 5, Malignant malaria. Intra-muscular injection of 18 grains of sulphate of quinine. Ten days later large quantity of pus evacuated. From 50 c.c. of the pus, 0.0012 gramme of quinine alkaloid was obtained.

No. 6, Malignant malaria. Intra-muscular injection of 10 grains of bi-hydrochloride of quinine. Death 26 hours later. Amount of quinine alkaloid recovered from muscle, 0.041 gramme.

<sup>1</sup> The oral method of administration of quinine is greatly neglected by some medical officers who are placed in charge of cases of malarial fever.

No. 7, Malignant malaria. Intra-muscular injection of 20 grains of bi-hydrochloride of quinine. Death 2 hours later. Amount of quinine alkaloid recovered from muscle, 0.344 gramme.

No. 8, Lobar pneumonia, Pneumococcal meningitis. Intra-muscular injection of 15 grains of bi-hydrochloride of quinine. Death 20 hours later. Amount of quinine alkaloid recovered from muscles, 0.0215 gramme.

In every instance the quinine was injected into the muscles in concentrated solution—the total quantity of fluid injected did not amount to more than a few c.c. The results show, however, that the absorption was rapid, as only traces of quinine were recovered from the entire lesions, apart from a small focus which was reserved for microscopy, and from the surrounding muscular tissue. In No. 7, 20 grains of the bi-hydrochloride of quinine were injected into the muscles, and the patient died two hours later, but only 0.3 gramme was recovered. In each instance the area of necrosis was considerable, and the tissues showed very marked changes on microscopical examination.

#### AN EXAMINATION OF MUSCLE TISSUE NECROSSED FROM QUININE INJECTIONS FOR THE PRESENCE OF MALARIAL PARASITES.

Certain specimens of muscular tissue which had been examined for the presence of malarial parasites subsequent to the injections of quinine showed most unexpected results. The blood cells in the necrotic vessels were completely haemolysed, yet malarial parasites were present in some instances in relatively large numbers and the bodies of the parasites were well stained. The parasites would be diminished in numbers as compared with the control muscle from another area of the body or from some visceral lesions, but perfectly staining parasites were found lying in a necrosed vessel in which there were no normal red cells. It is strong evidence that destruction of red cells and tissues generally is much more readily excited by quinine solutions than malarial parasites.

One case will be referred to in detail to emphasize this fact:

##### *Malignant Malaria.*

Blood film showed a very heavy infection of red cells with the ring form of parasites. Sporulating parasites were also numerous. Time 10.30 a.m.

20 grains of bi-hydrochloride of quinine were injected intra-muscularly at 11 a.m. and 20 grains were injected intra-venously. Death at 3.45 p.m. on same day.

Wide areas of necrosis of muscle at the seat of the quinine injection existed. There was great destruction of the walls of blood vessels and the blood cells were completely lysed, while the muscle tissue itself showed considerable necrosis. Large dot parasites with well-stained bodies and abundance of pigment were relatively numerous lying among the red cell *débris*. Parasites

however were far more numerous in the internal organs and in other muscular areas.

#### INTRA-MUSCULAR INJECTION INTO FROGS.

Several frogs received intra-muscular injections of quinine bi-hydrochloride in saline in suitable doses for body weight, and were killed three hours later. The muscles at the sites of the quinine injections were opaque. There was marked oedema, complete necrosis of muscle fibres, other fibres vacuolated or represented as granular masses and haemolysis of red cells in the affected areas.

Four frogs with an average weight of 60 grammes were injected (i.m.) with quinine and the resulting lesions were as follows:

1. Frog injected with 0.0004 gramme of quinine bi-hydrochloride (0.1 c.c.) and killed 22 hours later. Muscles opaque and showed extensive necrosis.

2. Frog injected with 0.0004 gramme of alkaloid in 0.02 c.c. of ether and killed 22 hours later. Extensive muscular necrosis. Slight inflammatory reaction.

3. Quinine injection as in case of frog 1. Animal killed 3 days later. Extensive muscular necrosis—abundant haemorrhages—and widespread inflammation were very evident.

4. Frog injected with 0.0004 gramme of the alkaloid suspended in saline and killed 3 days later. Results similar to Experiment 3.

These results were similar to those obtained in the case of the warm-blooded animals.

#### CONCLUSIONS.

- (1) Concentrated preparations of quinine produce more intense necrosis than dilute, but dilute preparations such as are of practical utility excite oedema and necrosis at the seat of inoculation. The difference between these two methods of quinine inoculation is not of sufficient value to justify active opposition to the method commonly employed.

Inoculation of quinine in solutions so dilute as to avoid oedema and tissue necrosis is not of practical utility in the human subject.

- (2) A concentrated solution of quinine is absorbed rapidly from the tissues as shown by chemical analysis even in patients who are *in extremis*. It is not apparently stored as such in liver, kidneys, or heart muscle.

- (3) It is essential to realise that tissue necrosis—spreading oedema and local blood destruction—are produced by the solvents employed for quinine administration and the effects are only slightly inferior to those excited by quinine salts and the alkaloid.

- (4) No advantage was obtained by the addition of olive oil or fat or by injecting the alkaloid dissolved in alcohol, or ether, whether in concentrated or in a dilute solution.

- (5) Tissue necrosis occurs immediately and persists for a considerable period. In some instances the fibro-myositis which results is associated with

a fibro-neuritis which causes various symptoms definitely related to the pathological processes.

(6) Necrosis of blood vessels in the area of inoculation is a common result. This leads to small haemorrhages into the tissues, and has caused severe haemorrhages in the human subject, and experimentally, from rupture of a large vessel. The destruction of the vessel wall is associated with an accompanying thrombosis.

(7) An extensive necrosis produced by an intra-muscular injection of quinine, in the neighbourhood of an important nerve trunk, may result in nerve palsy. Experimentally, complete degeneration of the great sciatic and other nerves has been produced apart from any direct injury to the nerve at the time of the inoculation. In the human subject this disastrous result may be due to spreading oedema and extensive tissue necrosis.

(8) Experimentally, no leucocytosis has ever occurred from quinine injections; on the other hand a leucopenia may develop while an increase of large hyaline cells has been recorded on several occasions.

(9) No essential differences in the degree of tissue necrosis from intra-muscular injections of quinine in malarial fever or malarial fever associated with blackwater fever were observed.

(10) Repeated intra-muscular injections of quinine should not be given into the same area of muscle, or tissue directly adjacent, as otherwise permanent injury of muscle<sup>1</sup> or nerves may occur. •

#### ACKNOWLEDGMENTS.

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To Captain Moir, A.V.C., Bacteriologist to the A.V.C., B.S.F., I am indebted for considerable help in the investigation of the cast mules and horses which were inoculated with quinine.

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The tissue from human sources has been procured for me by various bacteriologists in the Command, to whom my thanks are due.

To Corporal P. Panichelli, M.S.M., R.A.M.C. (T.F.), I am indebted for the illustrations which serve to explain what the text is incapable of defining.

<sup>1</sup> The gluteal regions, obtained from a man who had daily intra-muscular injections of quinine, nine in all, were shown at the British Medical Association meeting in London, 1919. As a result of the injections wide tracts of muscle were necrosed and only fragments of healthy tissue remained.

## DESCRIPTION OF PLATE III.

- Fig. 1. *Quinine rabbit*, 53-(4). Section of muscle from a rabbit which had received an intra-muscular injection of 0.04 gramme of alkaloid quinine in 1 c.c. of alcohol four days before death.
- Fig. 2. Agglutination of red blood corpuscles in a large vessel in the tissue of a horse which had been injected with 1.1 gramme of bi-hydrochloride of quinine 30 hours previously.
- Fig. 3. Complete necrosis of large artery as a result of an intra-muscular injection of quinine alkaloid in brandy.
- Fig. 4. Section of great sciatic nerve in a rabbit which had received an intra-muscular injection of 1 grain of quinine alkaloid in alcohol 11 days previously.
- Fig. 5. Muscle from a mule which had received 2.25 grammes of bi-hydrochloride of quinine in 5 c.c. of saline five days before death. Natural size.
- A. Oedema, necrosis, patches of haemorrhage.
  - B. Haemorrhagic line showing intense inflammation.
  - C. Oedematous muscle.
- Fig. 6. Portion of human muscle from a case of Blackwater Fever and Malaria. Patient had received an intra-muscular injection of 20 grains of bi-hydrochloride of quinine 48 hours before death. Natural size.



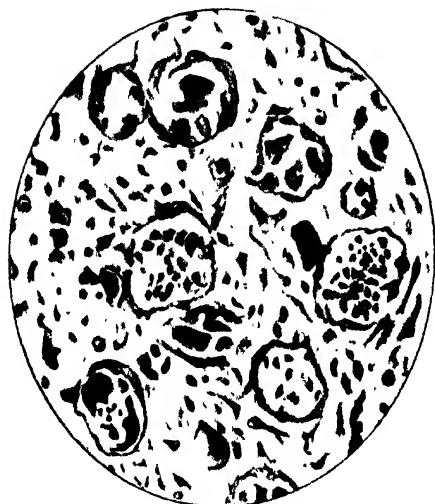


Fig. 1



Fig. 2



Fig. 3

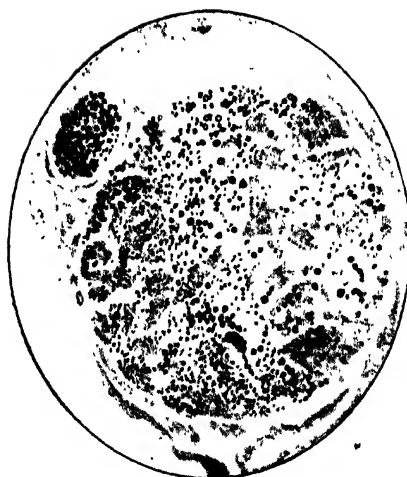


Fig. 4

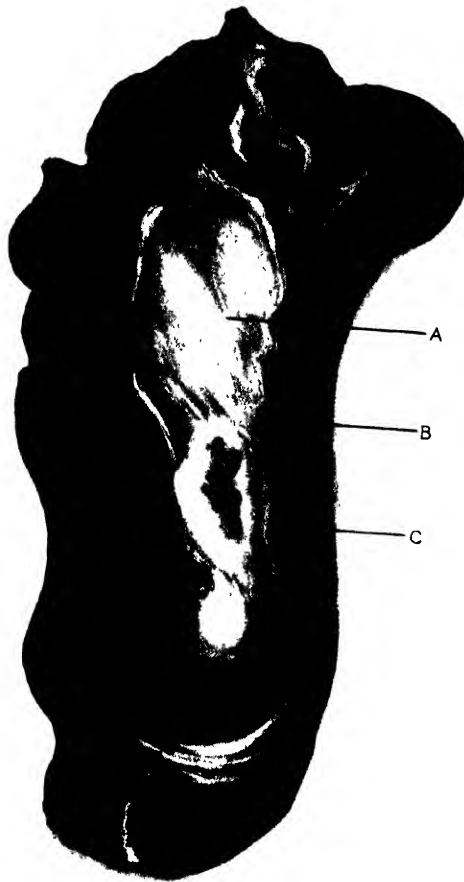


Fig. 5



Fig. 6





# STUDIES ON THE ACTION OF ELECTROLYTES ON BACTERIA.

## PART-I.

### THE ACTION OF MONOVALENT AND DIVALENT SALTS ON THE CONDUCTIVITY OF BACTERIAL EMULSIONS.

By C. SHEARER, F.R.S.

*(From the Pathological Laboratory, University of Cambridge.)*

(With 8 Charts.)

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#### I. INTRODUCTION.

To the bacteriologist the problem of how chemical substances enter and leave the cell presents many features of special interest. It is well known that singularly few bacteria produce true exotoxins, the majority forming endotoxins, but we possess little information as to the conditions under which these pass the cell-wall. In some instances it would seem that some form of autolysis enters in the matter to a considerable extent<sup>1</sup>.

In the case of the meningococcus, Gordon (1918) has pointed out that different strains of this germ possess widely different toxic powers for mice, but that they all have the same minimal lethal dose for these animals when ground up and extracted with distilled water. Thus in this case it would look as if toxicity depended on the ease with which the endotoxin diffuses out of the cell; while some races of the germ allow it to escape freely, others only permit it to pass in small quantities, but that all strains possess the same amount. Similar conditions probably hold for many other pathogenic bacteria.

The striking part played by capsule formation in the acquisition of virulence by many races of bacteria clearly points to the importance of this struc-

<sup>1</sup> These studies had their origin in an attempt to determine some of the conditions of autolysis in the meningococcus.

ture in preventing the penetration of many substances into the bacterial cell. Danysz (1900) has drawn attention to the fact that anthrax bacilli frequently possess arsenic and serum resistant qualities which are always coupled with their ability to form capsules. It has been shown that organisms like the pneumococcus and some streptococci, which sometimes acquire this power, are invariably most virulent in the capsulated condition. The capsule would seem to play a rôle similar to the action of one colloid on another, in preventing aggregation and flocculation, and the notorious difficulty always experienced in agglutinating these forms amply confirms this contention. This is supported by Porges's (1905) demonstration that they are easily agglutinable, if the capsules are first removed by heating a short time in weak acid.

It is clear that the question as to how endosmosis and exosmosis takes place in living bacteria is one of fundamental importance, and its solution is bound up with many questions of infection, virulence and immunity. It is remarkable that its investigation so far has attracted little attention from bacteriologists. If we turn to the domain of general physiology, we find a large and ever-growing literature dealing with the subject. It has been attacked from many sides, and the application to its study of some of the recent discoveries of colloid and physical chemistry have added very much to our knowledge, and our methods of dealing with the problem have been greatly extended and improved.

The question is necessarily a complex one, in which a large number of related and interdependent actions are simultaneously taking place. It involves the passage of substances from the external medium, through the cell-wall to the cytoplasm, across the intermediary boundary phases. In a balanced solution, that is, one that contains two or more salts in definite proportions, so that their specific individual action does not come into play, but is antagonised, living cells can remain for relatively long periods without suffering any injury. If the salts are sufficiently numerous, such solutions can even act as nutrient fluids, in which many plants and bacteria can actively grow and reproduce. It has been pointed out that under these conditions the cell-wall must be semi-permeable, that the passage through it of salts must be conditioned to a large extent by the chemical changes going on within the cell, and that the physical properties of the cell-wall play a minor part in the matter. Regarded in this light the problem is a dynamical one, in which equilibrium is constantly being adjusted between the external and the internal conditions.

If we place living cells, on the other hand, in weak but pure solutions of these salts we find we are dealing with a different problem. The fundamental conditions are now altered and the normal stability of the cell-wall has been destroyed. In such a solution we are studying the unantagonised action of a salt on the cytoplasm.

The results obtained from the investigation of this side of the question are of great experimental interest. It is particularly interesting to apply

experiments of this kind to bacteria, as there is no other class of organisms in which surface conditions and the properties of the cell-wall play a greater part, as they present relatively such a great extent of surface for a given mass, on account of their small size, surface conditions on all occasions determine their behaviour. The following paper for these reasons is mainly devoted to an investigation of the action of various salts in pure solutions in altering the cell-wall and surface conditions of bacteria.

It has been shown by Oker-Blom (1900), that if we estimate the relative conductivity of various mixtures of sand and a solution of NaCl, we find this is proportional to the quantity of sand in the solution. As an electric current is carried through a solution by its ions, the rate at which these will conduct the current is dependent on two factors, first the potential gradient, and secondly the friction or resistance offered by the solution to the passage of the ions. If the potential gradient is kept fixed, then any additional obstruction to the passage of the ions will necessarily increase the resistance and lower the conductivity. In a mixture of 61 parts quartz sand and 39 parts NaCl solution, he found the conductivity was 24.5 per cent. that of the NaCl solution without sand. The ions of such a mixture were forced by the grains of sand to take a zigzag course in passing from one electrode to another, and the resistance is proportionally increased.

In a similar manner the conductivity method has been used by Róth (1897), Bugarsky and Tangl (1897), Oker-Blom (1900), Stewart (1899), Woelfel (1908) to determine the proportion of blood corpuscles to plasma. If we measure the comparative conductivity of blood serum, whole blood, and corpuscles alone, we find the plasma has the highest figure, the corpuscles the lowest, and the whole blood an intermediate position. Thus these workers conclude, that the cellular elements of the blood, like the sand grains in the previous instance, offer considerable resistance to the passage of the ions of the plasma.

Stewart (1910), as the result of extensive studies on the conductivity of normal as compared with laked blood, comes to the conclusion that the cell "envelopes" of the corpuscles are relatively impermeable to the ions with which they are normally in contact. He found, moreover, that any strong cytolytic agent, such as saponin, which rapidly destroys the envelop membrane, at once increases the conductivity of the corpuscles.

This conclusion would seem to be supported by Höber's (1913) experiments, where he has shown that if a conducting body is placed in the axis of a coil of wire, through which a rapidly alternating current is being passed, it will dampen or diminish this current in proportion to its power of conductance. The internal conductivity of blood cells is therefore greater than their conductivity as determined in the ordinary way. This indicates that the cell-wall or plasma membrane of the corpuscles offers considerable resistance to the passage of ions. He also found that saponin, which undergoes no dissociation, has little effect on the internal conductivity, while its cytolytic

action on the cell-wall greatly increases conductivity as determined by the Kohlrausch method; thus confirming Stewart's previous discovery.

If instead of sand grains or blood cells we add living bacteria to a clear solution of Ringer's fluid, we find in a similar way, that the resistance will increase and the conductivity decrease in proportion to the number of bacteria added. If sufficient bacteria are added to turn the fluid a white milky colour, the resistance is usually double that of the clear solution; if we add enough bacteria to turn the mixture into a thick paste the resistance may be trebled, while if we centrifuge the germs down in a solid mass, the resistance of the bacterial deposit will now be five or six times that of the original plain fluid. By placing bacteria in a similar manner in various salt solutions, if we take the precaution to make these solutions of the same conductivity as the Ringer's solution, we can determine the specific action of these solutions in altering the normal conductivity of the bacterial cell as originally determined in the Ringer's solution.

The work of Osterhout (1913), McClendon (1910), and Gray (1916) has shown that the Kohlrausch conductivity method is readily applicable to the study of the action of salts, in this manner, on living plant tissues and the animal egg-cell. Moreover the work of Perrin (1904), Girard (1910), (1919 a), (1919 b), Mines (1911) and Brooks (1917), on the passage of electrolytes through artificial and natural membranes, form a series of researches of remarkable interest, when compared with the results obtained by the conductivity method.

The conductivity method seems to have been first employed in the investigation of living cells by a number of independent workers about the same time. Among these are Róth (1897), Bugarsky and Tangl (1897), and Stewart (1910). Stewart found as already mentioned that the conductivity of blood plasma was greater than that of the whole blood, and that the resistance rose rapidly with an increase in the number of corpuscles. The action of saponin, in lowering the resistance of the corpuscles, was noted and he drew attention to the fact that it produced this effect as well on dead corpuscles. McClendon (1910) was the first to apply the method, using special electrodes, to the estimation of the changes taking place in the conductivity of the Echinoderm egg on fertilisation. This work was elaborated still further by Gray (1916), who demonstrated the marked action of the trivalent salts in altering the conductivity of these eggs in sea-water. The method was applied about the same time independently by Osterhout (1915), to determine the conductivity of the tissues of the marine alga *Laminaria* to salts in pure and balanced solutions.

It was found difficult working with bacteria to obtain resistances as high, even with the thickest emulsions or solid masses of bacteria, as those obtained by these workers. Osterhout (1918) using a special apparatus, and placing a large number of discs of *Laminaria* tissue one against another, like a roll of coins, was able to obtain resistances well over a thousand ohms; while Gray, using eggs that had been specially washed to remove the jelly-like

outer membrane, obtained resistances of 250–350 ohms, while the resistance of the same quantity of sea-water under the same conditions was only 16 ohms.

## II. DESCRIPTION OF EXPERIMENTS.

It was found possible, using bacteria made up into thick emulsions, to obtain very similar consecutive readings of their resistances, if after three or four preliminary washings in Ringer's fluid they were centrifuged down into a solid mass, and then made up in a thick paste with the same quantity of fresh fluid each time. In the following experiment (Table I) is given a

Table I.

Experiment giving a series of consecutive readings of resistances made on the same meningococcus emulsion, in Ringer's solution. The bacteria were centrifuged down into a solid mass each time and then made into a thick paste with the same quantity of Ringer's sol. Temp. 25° C. Cell constant =  $4.22 \times 10^{-1}$ .

1st time	104 ohms resistance	6th time	108 ohms resistance
2nd	106	7th	108
3rd	105	8th	107
4th	106	9th	107
5th	108	10th	108

series of ten consecutive readings taken in this way, on the same emulsion of the meningococcus. It will be seen that they agree with one another very well, and only differ within a margin of a few ohms. There is a slight tendency for the resistance to rise slightly towards the end of the experiment. This is doubtless due to the washing away of salts brought over with the germs, despite the preliminary washings from the culture medium. It can be neglected as it is always slight, and the resistances for the most part in the following experiments have to deal with a fall not a rise. Only fresh 24 hrs. cultures were used, 24–30 plates of tryptagar being sufficient to furnish enough material for one experiment. The bacteria were washed off the plates in a large quantity of neutral Ringer's solution<sup>1</sup> and centrifuged down and rewashed three times in succession in considerable quantities of fresh fluid each time, before being used for any of the experiments as already mentioned.

All measurements were made in a thermostat tank, at a fixed temperature of 25° C., which did not vary more than a twentieth of a degree. A direct reading Kohlrausch bridge was employed. In the earlier experiments the resistances were determined with a large Hamburger cell. This was made to fit into the tubes of the centrifuge direct and the emulsions were centrifuged down in the conductivity cell itself. In this way it was possible to get particularly thick emulsions having about 110 ohms resistance, while the same quantity of Ringer's solution had only 26 ohms resistance. In the later experiments a much smaller cell was employed and the emulsions were made

<sup>1</sup> M/8 KCl	...	25 c.c.	0.0031 M.	0.024 % KCl.
CaCl <sub>2</sub>	...	15	0.00187 M.	0.0208 % CaCl <sub>2</sub> .
NaCl to	...	1000	0.12 M.	0.7 % NaCl.

considerably thinner, which gave slightly more uniform readings, and also saved time in the preparation of the material itself. In this case resistances of 150–200 ohms could be obtained, while the same quantity of Ringer's solution had about 85 ohms resistance. The cell constants for these cells are given in the tables giving the data of the various experiments<sup>1</sup>.

If sufficient care was taken to get the original emulsion fairly thick, resistances of 110 ohms could be pretty constantly obtained with the meningococcus and slightly higher resistances with *B. coli*. The same quantity of Ringer's solution in the same cell under the same conditions of temperature having about 26·7–37 ohms resistance. Thus about three-quarters (or a little less) of the resistance of the above solution is due to the presence of the living bacteria. It was found that dead bacteria offer no resistance to the passage of the ions of a solution. Emulsions of dead bacteria have almost the same resistance as that of the fluids in which they are suspended. If the above emulsion of bacteria in Ringer's solution were killed by adding a drop of formalin or warming the emulsion to 55° C. for a few minutes, then on washing the emulsion several times in fresh changes of Ringer's solution to get rid of the formalin or of any salts derived from the dead bacteria, it will be found that the resistance has fallen from 104 or 108 ohms to 26·7 or 30 ohms, the resistance of the Ringer's solution alone. Thus there would seem to be something about the living condition which produces the resistance, and that dead cells offer no more than would so much agar or gelatine.

Living bacterial emulsions undergo little change in conductivity on standing for several days in Ringer's solution, showing a slight tendency to fall on account of adsorption of a little of the salts by the bacteria; this fall is always slight.

If, however, we make up the bacterial emulsion in pure NaCl\* instead of Ringer's solution, the NaCl employed having the same conductivity as that of the Ringer's solution, *i.e.* one in which the resistance is 26·7 ohms (which is not very far removed in strength from a 0·85 per cent. NaCl solution or 0·124 M.), we obtain as in the case of the same emulsion in Ringer's solution an initial resistance of 110 ohms. This gradually drops within a short time and at the end of 30 or 40 minutes becomes the same as that of the NaCl solution without bacteria, *i.e.* 26·7 ohms. (See Table II A and Curve I.)

Thus pure NaCl of about the same concentration as that present in the blood gradually destroys the resistance offered by the bacterial cell. If the germs are allowed to lie in the NaCl for several hours, it will be found that at the end of this time they are dead. If they only remain in the NaCl a short

<sup>1</sup> Unfortunately in the case of the large cell this was not determined until most of the experiments had been made and the electrodes had been damaged by a slight accident. The cell constant was determined after the cell had been repaired and only applies approximately to this cell.

\* All solutions used in the following experiments were made from water that had been distilled three times from glass, and Kahlbaum salts were employed.

time, and are then transferred to Ringer's solution again, they immediately regain their normal resistance and suffer no injury.

In the case of *B. coli* even at the end of one hour in NaCl they are seen to be actively motile, and death only takes place slowly after some time, as the probable result of the gradual diffusion out of the cell of some of the essential chemical substances necessary to the living state.

Table II A.

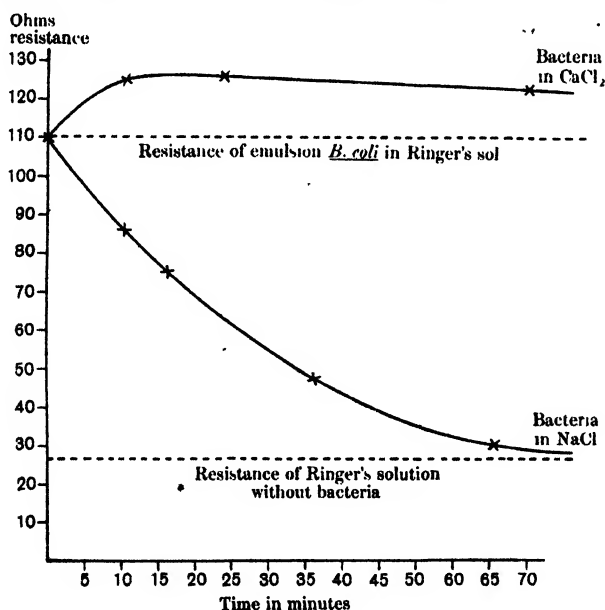
Sodium chloride Experiment.

Temp. 25° C. Resistance constant of cell =  $4.22 \times 10^{-1}$ .

1. Resistance of Ringer's and NaCl (0.124 M.) each	...	26.7 ohms.
2. Resistance of Type III Meningococcus emulsion in Ringer	110 ohms.	
3. Resistance due to bacteria, 110 - 26.7	...	83.3 ohms.
4. Resistance of same emulsion in NaCl of the same conductivity as Ringer's sol. after		
	10 min.	90 ohms.
	15	80
	35	49
	65	28

At the end of the experiment the emulsion was subcultured, at the end of 48 hrs. little growth had taken place, showing the emulsion at the time of subculture was practically dead.

Curve I, showing the resistance of emulsion of *B. coli* in pure  $\text{CaCl}_2$  and NaCl solution of the same conductivity as neutral Ringer's solution. Temp. 25° C. Resistance of all solutions 26.7 ohms. Cell constant =  $4.22 \times 10^{-1}$ .  $\text{CaCl}_2 = 0.1 \text{ M.}$ ,  $\text{NaCl} = 0.124 \text{ M.}$



If, when the resistance of the bacterial emulsion has fallen in the NaCl solution, a little trace of  $\text{CaCl}_2$  is added, it again returns to its normal conductivity, and suffers no injury. Thus the  $\text{CaCl}_2$  antagonises the action of the NaCl.



It was found that a small trace of  $\text{SrCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{CdCl}_2$  could also antagonise in a similar manner the action of  $\text{NaCl}$ . (See Tables and Curves of these experiments.)

Table II B.

Calcium chloride Experiment I. Temp. 25° C.			
Cell constant = $4.22 \times 10^{-1}$ .			
1.	Resistance of Ringer's and $\text{CaCl}_2$ (0.1 M.) <sup>1</sup> each	...	26.7 ohms.
2.	Resistance of emulsion of <i>B. coli</i> in Ringer's sol	...	120.0
3.	Resistance due to bacteria, 120 - 26.7	...	93.3
4.	Resistance of same emulsion washed in 3 changes of $\text{CaCl}_2$ of the same conductivity as Ringer's sol. after		
	10 min.	...	124 ohms.
	25	...	124
	2 hrs.	...	120

Emulsion grew well on subculture at end of experiment.

Table III.

Calcium chloride Experiment II. Temp. 25° C.			
Cell constant = $9.5 \times 10^{-1}$ .			
1.	Resistance of Ringer and $\text{CaCl}_2$ solutions each	...	85.0 ohms.
2.	Resistance of emulsion of <i>B. coli</i> in Ringer	...	135.0
3.	Resistance due to bacteria, 135 - 85	...	50.0
4.	Resistance of same emulsion in $\text{CaCl}_2$ sol. (0.1 M.) after		
	25 min.	...	167 ohms.
	30	...	161
	35	...	161
	2 hrs.	...	161

Emulsion grew well at end of experiment on subculture.

Table IV.

Calcium nitrate Experiment. Temp. 25° C.			
Cell constant = $9.5 \times 10^{-1}$ .			
1.	Resistance of Ringer and calcium nitrate sols. each	...	85.0 ohms.
2.	Resistance of emulsion of <i>B. coli</i> in Ringer	...	120.0
3.	Resistance due to bacteria, 120 - 85	...	35.0
4.	Resistance of same emulsion in $\text{CaNO}_3$ isotonic with Ringer	...	124.0
5.	Resistance of same emulsion in $\text{CaNO}_3$ after 1 hour	...	123.0

Emulsion grew well at end of experiment on subculture.

Further experiments with other salts of the monovalent group, such as  $\text{KCl}$ ,  $\text{LiCl}$ ,  $\text{RbCl}$ ,  $\text{CsCl}$ , showed that each of these in turn possessed in a varying degree the power of reducing the resistance offered to the passage of the ions of a solution, in a manner similar to  $\text{NaCl}$ . In all instances this increase of conductivity was reversible, the bacteria returning to their normal condition on being transferred to any balanced solution, such as Ringer's, sea-water, or van't Hoff's solution. The addition to any of the above monovalent solutions of a slight trace of a divalent salt such as  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ , or  $\text{BaCl}_2$ ,  $\text{CdCl}_2$ , prevents the fall in resistance in the monovalent solution from taking place.

<sup>1</sup> The molecular strengths of the solutions have only been roughly calculated from the conductivity

Table V.

Lithium chloride Experiment. Emulsion of *B. coli*. Temp. 25° C.  
Cell constant =  $4.22 \times 10^{-1}$ .

1. Resistance of Ringer and LiCl (0.195 M.) each	...	...	26.7 ohms.
2. Resistance of <i>B. coli</i> emulsion in Ringer's sol	...	...	147.0
3. Resistance due to bacteria, 147-26.7	...	...	120.3
4. Resistance of same emulsion in LiCl sol. (0.95 M.) after 25 min.	...	...	113.0
5. Resistance of same emulsion in LiCl sol. (0.95 M.) after 35 min.	...	...	100.0
6. Resistance of same emulsion in LiCl sol. (0.95 M.) after 50 min.	...	...	86.0

Table VI.

Rubidium chloride Experiment. Emulsion of *B. coli*. Temp. 25° C.  
Cell constant =  $9.5 \times 10^{-1}$ .

1. Resistance of Ringer and RbCl	...	...	85 ohms.
2. Resistance of emulsion of <i>B. coli</i> in Ringer	...	...	120
3. Resistance due to bacteria, 120-85	...	...	35
4. Resistance in RbCl sol. after 40 min.	...	...	105
5. Resistance in RbCl sol. after 50 min.	...	...	100

Table VII.

Hydrochloric acid Experiment. Emulsion of *B. coli*. Temp. 25° C.  
Cell constant =  $9.5 \times 10^{-1}$ .

1. Resistance of Ringer and HCl (0.049 M.) each	...	...	85 ohms
2. Resistance of emulsion of <i>B. coli</i> in Ringer	...	...	110
3. Resistance of same emulsion in HCl after 45 min.	...	...	128
4. Resistance of same emulsion in HCl after 65 min.	...	...	126

It was possible to transfer the same emulsion from Ringer to NaCl a number of times in succession, and get a fall each time in NaCl and a return to the normal resistance in Ringer. (See Curve II.)

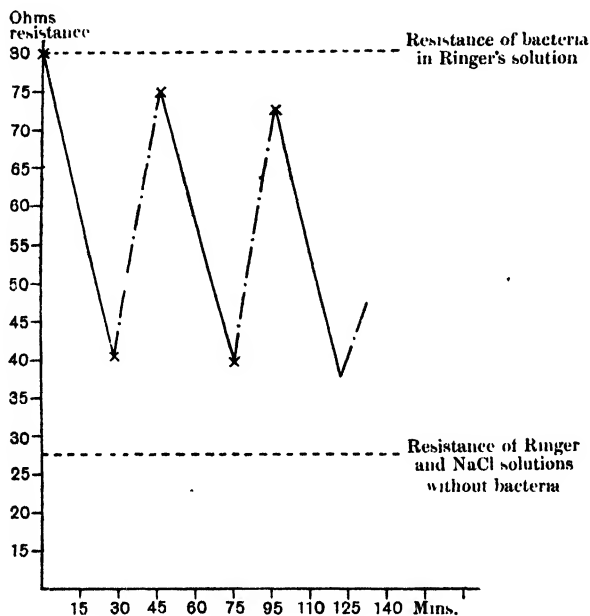
Of the members of the monovalent group, an exception must be made in the case of the H ion. A rapid rise in resistance takes place with all emulsions of bacteria placed in weak solutions of HCl, of the same conductivity as Ringer's solution; as will be seen on consulting the Table and Curve dealing with this experiment.

If, on the other hand, a bacterial emulsion is made up in a solution of some bivalent chloride, such as  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ ,  $\text{BaCl}_2$ <sup>1</sup>, of the same conductivity as Ringer's solution, if the emulsions are sufficiently thick to give fairly high resistances, a slight rise in resistance usually takes place. This is followed after 6 or 9 hours by a slow fall. If, after 12 or 15 hours when this fall is well marked, the emulsion is returned to Ringer's solution, van't Hoff's solution, sea-water, or any balanced solution, it will be found that the emulsion does not regain its normal conductivity, as when a little  $\text{CaCl}_2$  is added to a monovalent salt. There is this difference between the fall of resistance of an emulsion in a monovalent and a divalent salt, that in the latter the fall is irreversible.

<sup>1</sup> In the  $\text{CaCl}_2$  and other bivalent salt solutions the emulsions are always more viscid and less fluid than in the monovalent salt solutions.

Curve II. Experiment showing the antagonistic action of  $\text{CaCl}_2$  in Ringer's solution to the action of pure NaCl solution of the same conductivity as Ringer's solution. Meningococcus emulsion. Temp.  $25^\circ \text{C}$ . Unbroken lines represent the meningococcus in NaCl solution, broken lines—same emulsion in Ringer's sol., the emulsion being transferred three times from NaCl to Ringer's sol. In each instance that the emulsion was placed in NaCl the resistance fell to rise again almost to the normal when transferred back to Ringer.

A similar curve was obtained when a pure  $\text{CaCl}_2$  solution was used of the same conductivity.  $\text{NaCl} = 0.124 \text{ M}$ .



If, in place of living, we use an emulsion of dead bacteria for any of the foregoing experiments, none of these changes take place. They are therefore dependent on the cells of the emulsion being alive, as already mentioned; dead bacteria offer no resistance to the passage of the ions of a solution. The gradual fall of resistance which is irreversible in a bivalent chloride is the result of the slow death of the emulsion.

It may be suggested that these effects are to some extent the result of injury to the cell by the electric current or its forcing the ions of the solution through the cells. This cannot be the case, as the action of the monovalent cation is as marked, when the bacteria are placed in these solutions in the absence of any electrical current, as when a current is passing.

In a previous paper (1917) I have demonstrated that the meningococcus is rapidly killed if allowed to remain in 0.85 per cent. NaCl solution for a short time, and that this toxic effect is avoided if a small trace of  $\text{CaCl}_2$  is added to the solution. Thus in the absence of any current we can get the same results as when a current is employed. There is a large amount of evidence to show

that the characteristic action of a monovalent as compared with a bivalent salt in pure solutions is universal for all living cells.

Loeb (1906) has described similar effects of the action of dilute NaCl on the egg of the marine teleost *Fundulus*. The eggs of this fish develop normally in sea-water. If they are put into pure NaCl having the same concentration as that of the sea-water, none of them develop. If, however, a trace of  $\text{CaCl}_2$  is added, as many eggs develop as in ordinary sea-water. The same effect is produced if a little Sr, or Ba, is added in place of the  $\text{CaCl}_2$ . He has also shown (1906) that muscle tissue contracts rhythmically when immersed in pure solutions of salts with a monovalent cat-ion, such as Na, Li, Rb and Cs, but that the addition of a small quantity of a bivalent cat-ion inhibits these contractions.

The interest of the foregoing results consists in that they show that the action of electrolytes on bacteria is similar in all respects to their action on plant and animal cells, as determined in different ways by Loeb and Wastenays (1915), Osterhout (1915), Gray (1916), McClendon (1910), Brooks (1917), and other workers.

In *Laminaria*, Osterhout (1915) finds that with  $\text{CaCl}_2$ , and also with  $\text{BaCl}_2$  and  $\text{SrCl}_2$ , there is invariably a brief temporary rise of resistance when placed in these solutions of the same conductivity as that of the sea-water employed in his experiments. This is followed by a gradual fall. On account of the low resistance used in the present experiments, this preliminary rise of resistance is not so marked. Its presence was frequently demonstrated with  $\text{CaCl}_2$  and  $\text{SrCl}_2$ , as the data and curves of some of the experiments given in this paper plainly show. In the few experiments done with  $\text{BaCl}_2$ , it was not noticed, but it is clear that this salt can obviously antagonise the action of NaCl, and it undoubtedly belongs to the same group, and probably does not differ from them in this respect.

Table VIII.

Magnesium chloride Experiment.			
Temp. 25° C. Cell constant $-4.22 \times 10^{-1}$ .			
1. Resistance of Ringer and $\text{MgCl}_2$ solutions each	...	...	26.7 ohms.
2. Resistance of <i>B. coli</i> in Ringer's sol.	...	...	108.0
3. Resistance due to bacteria, 108-26.7	...	...	81.3
4. Resistance of same emulsion in $\text{MgCl}_2$ sol. (0.09 M.) after			
10 min.	...	100 ohms resistance	.
20	...	88	
25	...	80	
35	...	72	
45	...	72	

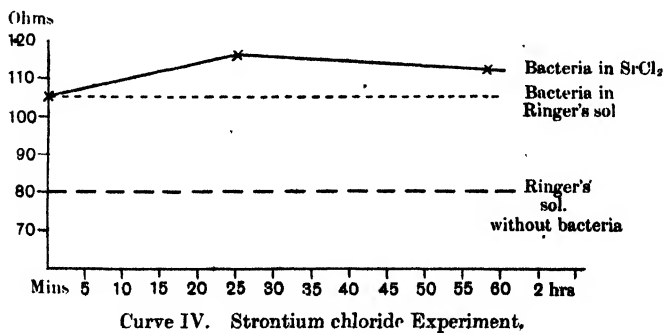
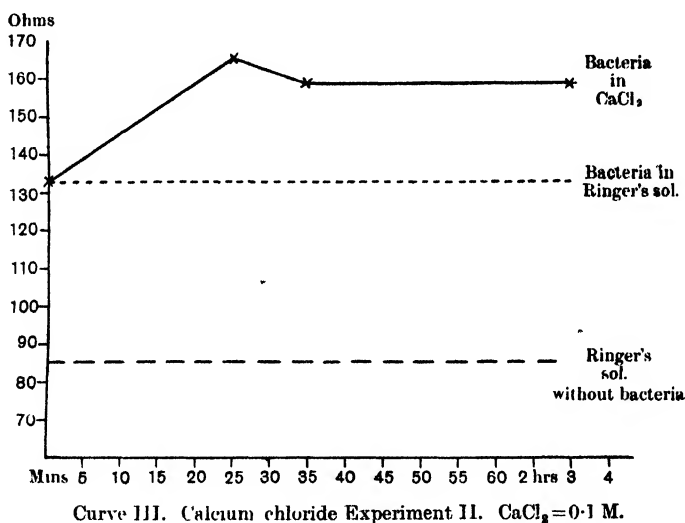
1 drop 40 per cent. formalin added. Resistance fell to 28 ohms.

In the light of the present experiments, the time-honoured practice of suspending living bacteria in normal physiological saline (NaCl 0.85 per cent., 29/200 or 0.145 M.) would have little to recommend it, beyond the ease with which most bacteria can be emulsified in such a solution.

## *Action of Electrolytes on Bacteria*

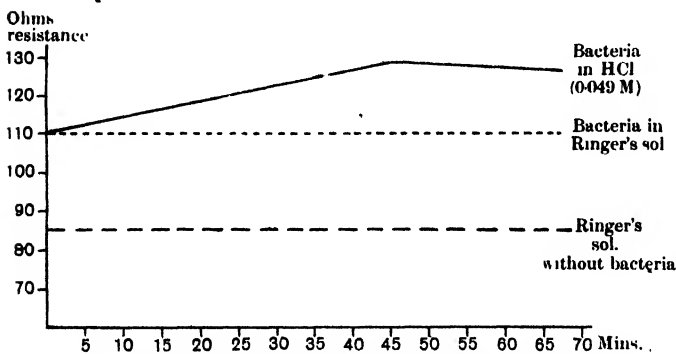
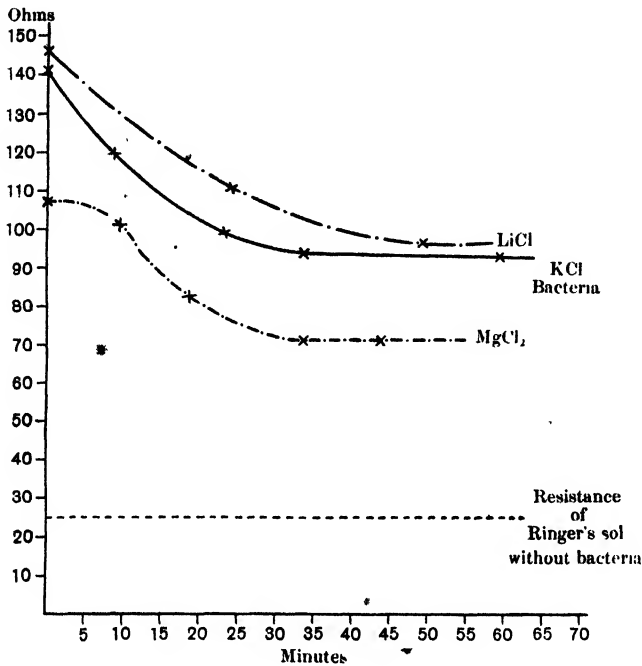
As usually employed in practical bacteriology, the action of the NaCl seldom really comes into play, as sufficient  $\text{CaCl}_2$  is always brought over from the culture medium to completely antagonise the NaCl. In Ringer's solution for instance the amount of  $\text{CaCl}_2$  present is only 0.00187 M. Thus in an ordinary bacterial suspension, unless this has been washed once with saline, sufficient  $\text{CaCl}_2$  is present to prevent the specific action of the NaCl. This is doubtless the reason why the injurious action of this salt in ordinary bacteriological manipulation has been so rarely noticed, Flexner (1907) in his classical paper on the meningococcus being one of the few to draw attention to it.

The use of saline solution in practical bacteriological work seems to have originated in a somewhat mistaken opinion of the importance of the rôle of osmotic pressure with regard to bacteria, for which the well-known paper of Fischer (1895), on the plasmolysis, is largely to blame. In distinction to red blood cells, bacteria can easily withstand wide changes in osmotic pressure. In the case of the meningococcus I have shown that this germ can readily survive 24 hours in pure glass distilled water. This is otherwise if the water



has been obtained from a metal still. Osterhout (1913) has also drawn attention to the fact that glass distilled water has no toxic action on *Spirogyra*, while metal distilled water has a marked action in this respect.

Curve V, showing the resistance of *B. coli* in Potassium chloride (=0.09 M.) solution.  
 $\text{LiCl}=0.195 \text{ M.}$   $\text{MgCl}_2=0.09 \text{ M.}$



Curve VI. Hydrochloric acid Experiment.

In regard to the action of  $\text{MgCl}_2$ , Osterhout (1915) found that a close study of the action of this salt revealed the fact that, although the fall due to its toxic action was very abrupt, resembling at first sight that of  $\text{NaCl}$ , this salt really acts like  $\text{CaCl}_2$ ,  $\text{BaCl}_2$  and  $\text{SrCl}_2$ , in that it produces a short temporary

rise in resistance, followed by an irreversible slow fall in resistance. If this should be the case then the fall in resistance obtained with bacteria should be irreversible. The following experiment would seem to show that this is the case, and that  $\text{MgCl}_2$  in its action is to be classed with the other bivalent chlorides as  $\text{BaCl}_2$ ,  $\text{SrCl}_2$ , and  $\text{MnCl}_2$ .

Table IX.

Experiment Demonstrating the Antagonistic Action of  $\text{BaCl}_2$  to  $\text{NaCl}$ . Temp.  $25^\circ \text{C}$ .

(a) To 100 c.c.  $\text{NaCl}$  of the same conductivity as Ringer's sol. a few crystals of  $\text{BaCl}_2$  were added until the resistance was 20 ohms.

(b) To 100 c.c. Ringer's sol. distilled water was added till the resistance equalled 20 ohms.

1. Resistance of *B. coli* emulsion in (b) was 95 ohms.
2. Resistance due to presence of bacteria was 75 ohms.
3. Resistance of same emulsion of *B. coli* (a) after 15 min. was 95 ohms.: practically no change.

Table X.

Irreversible action of  $\text{MgCl}_2$  on bacteria.

Temp.  $25^\circ \text{C}$ . Cell constant =  $9.5 \times 10^{-1}$ .

1. Resistance of Ringer and $\text{MgCl}_2$ solutions each ... ..	83 ohms.
2. Resistance of emulsion of <i>B. coli</i> in Ringer ... ..	138
3. Resistance due to bacteria, $138 - 83$ .. ..	55
4. Resistance in $\text{MgCl}_2$ sol. after 25 min. ... ..	96
5. Resistance of above emulsion plus 5 drops $\text{CaCl}_2$ (0.1 M.)	88
6. Resistance of above emulsion washed 3 times in fresh Ringer	88

Table XI.

Cadmium chloride Experiment.

Temp.  $25^\circ \text{C}$ . Cell constant =  $9.5 \times 10^{-1}$ .

1. Resistance of Ringer and $\text{CdCl}_2$ solutions each .. ..	85 ohms.
2. Resistance of emulsion of <i>B. coli</i> in Ringer ... ..	115
3. Resistance of emulsion of <i>B. coli</i> in $\text{CdCl}_2$ solution (0.065 M.)	116

Emulsion actively motile at the end of the experiment.

Table XII.

Strontium chloride Experiment.

Temp.  $25^\circ \text{C}$ . Cell constant =  $9.5 \times 10^{-1}$ .

1. Resistance of Ringer and $\text{SrCl}_2$ ... ..	80 ohms
2. Resistance of emulsion of <i>B. coli</i> in Ringer's sol. ...	105
3. Resistance of same emulsion in $\text{SrCl}_2$ sol. after	
25 mins. ... ..	115 ohms.
60 ... ..	112

In view of the results obtained in the foregoing experiments of the action of the monovalent and divalent salts in altering the normal conductivity of the bacterial cell, it is highly important to determine if these changes are correlated in any way with a modification of pathogenic power. To determine this fresh cultures of *B. anthracis* and the pneumococcus were used. The pneumococcus strain was passed through a number of mice before being used.

Emulsions of these bacteria were treated as in the preceding experiments, with NaCl, CaCl<sub>2</sub> and Ringer's solution, and injected into mice and the effects of these solutions on pathogenetic powers observed. In all instances the salt solutions used in these experiments were carefully sterilized before being employed for making up the emulsions of bacteria. The results of these injections are shown in Tables XIV and XV.

Thus a fresh culture of *B. anthracis* was made up in an emulsion in Ringer's solution, its resistance determined, and then transferred to NaCl solution of the same conductivity as the Ringer's solution, being rapidly washed in several changes of NaCl solution to eliminate all trace of the Ringer's solution. It was allowed to remain in the NaCl solution (0.124 M.) for about fifteen minutes, till its resistance had fallen about two-thirds of the way to that of the NaCl solution alone without bacteria, and an estimated dose was then injected into several or more mice. A similar dose of the same emulsion, which had remained in Ringer's solution all the time, was injected into a second lot of mice, of approximately the same weight as those of the first batch. Into a third group of mice a similar dose of the same emulsion was injected in NaCl solution, to which a trace of CaCl<sub>2</sub> had been added. All these emulsions were diluted down in their respective fluids, before being injected, so they rendered these fluids slightly turbid. The results obtained from experiments of this kind were invariably uniform.

It was found that when the resistance had fallen in the NaCl solution, the bacteria failed to kill the mice, or only did so after seven or eight days' time, while those mice that had received the same emulsion in a similar dose in Ringer's solution were sometimes dead within 18 hours and were always dead within 24 or 36 hours. The same applies to the mice that had received the germs in NaCl, to which a little CaCl<sub>2</sub> had been added. (See Tables XIV and XV.)

It would seem that either the majority of the bacteria in the NaCl solution were dead, when they were injected into the animals, or that the action of the NaCl was such as to render them almost harmless. Similar results were obtained when special care was taken to see that the resistance of the germs in the NaCl had not fallen too low. Subcultures made from these emulsions, at the time they were injected, gave a very good growth after 24 hours' incubation, showing that a fair percentage of the bacilli were still alive. There was therefore some evidence for thinking that the NaCl had a detoxicating action on the germs.

It was found when some of these bacilli were exposed in NaCl solution (0.124 M.) to the action of washed leucocytes, they were taken up by these leucocytes immediately, and within a short time few free bacteria remained outside the phagocytes. The same bacteria in Ringer's solution under similar conditions were taken up at a much slower rate by the leucocytes. This result again may be due to the detoxicating action of the NaCl on the bacteria, rendering them more liable to attack by the leucocytes.



If on the other hand we inject the same bacteria in similar doses in  $\text{CaCl}_2$  solution (0.1 M.) they kill the mice in even smaller doses than when they are injected in Ringer's solution. In such a solution the preceding experiments show that a slight rise of resistance takes place. It was found however that the  $\text{CaCl}_2$  solution itself without any bacteria often had an ill effect. In this respect  $\text{CaNO}_3$  seemed more toxic than  $\text{CaCl}_2$ . If two lots of mice were injected, one with a lethal dose of *B. anthracis* in  $\text{CaNO}_3$  and the other with the same germ in  $\text{CaCl}_2$ , the batch that had received the bacteria in  $\text{CaNO}_3$  always were the first to die. In making experiments with  $\text{CaCl}_2$ , control mice were always injected with some of  $\text{CaCl}_2$  alone without bacteria; it was seldom that these showed any ill effects from the dose of Ca employed in the previous injections. It is clear that in the  $\text{CaCl}_2$  solutions no detoxication takes place if the bacteria are injected after standing in this solution for 30 minutes, and possibly a slight increase of toxicity takes place.

### III DISCUSSION OF THE BEARING OF SOME OF THE RESULTS OF THE FOREGOING EXPERIMENTS ON THE RÔLE OF CERTAIN SALTS IN THE QUESTION OF WOUND INFECTION.

These results have a direct bearing on some recent research on wound infection and gas gangrene.

Bullock and Cramer (1919) find that the organisms of gas gangrene, when emulsified in saline ( $\text{NaCl}$  0.85 per cent. or 0.145 M.) and washed in several changes of this fluid to remove toxins, fail to kill mice and guinea-pigs, while similar doses of broth cultures of these organisms produce a violent gas gangrene which kills the animals in twenty-four hours. In washing the bacteria in several changes of  $\text{NaCl}$  to eliminate all toxins, it will be seen that they are repeating the conditions of the preceding experiments with this salt, where the  $\text{NaCl}$  destroys the normal stability of the cell wall and cytoplasm, which has been shown to be correlated by experiments on animals with a loss of toxicity. They find further that this action can be prevented by adding a little Ca salt, either to the culture in saline when it is being injected, or by injecting the animal a short time after with a large dose of Ca. In this case they are simply antagonising the action of the  $\text{NaCl}$ , and under these conditions, as has been shown in the previous experiments, the conductivity of the bacterial cell returns to its normal condition, and the germs regain their toxic power.

Unfortunately, to determine if the action of the Ca salts is on the bacteria or the tissues of the animal into which the bacteria have been injected, they incubated their cultures for three hours in weak  $\text{CaCl}_2$ ; "the suspension was then centrifuged, and the bacteria after washing with saline suspended in  $\text{NaCl}$ , and the suspension then injected into mice" (p. 521). As would be expected they find that after this treatment the germs are still non-toxic. They conclude from this that the action of the  $\text{CaCl}_2$  is not on the bacteria, and therefore must be on the tissues of the animal.

To this action they give the name of "kataphylaxis." It would seem, however, that the real point in question has been overlooked and this is the action of the NaCl on the bacteria. In testing the action of the  $\text{CaCl}_2$  on the germs, they make the mistake of washing this away with NaCl. In washing once in NaCl and resuspending the bacteria in this solution for injection, they have removed all trace of  $\text{CaCl}_2$ , and the unantagonised action of the NaCl again comes into action. The germs are now in the same state as they were previous to treatment with  $\text{CaCl}_2$ , and are unable to kill the animals.

No doubt the injection of the NaCl or the  $\text{CaCl}_2$  may lead to a certain amount of injury to the tissues at the local point of injection, due to the action of these salts on the tissue cells, but this action is bound to be as great on the bacteria, unless we assume there is a selective action on the tissue cells. Where the  $\text{CaCl}_2$  is added to the emulsion of bacteria in saline before injection, this cannot take place as a balanced solution results. It is obvious that the action of the salts on the tissues is not the controlling factor, as the result is the same in both cases.

In the light of the previous experiments, Bullock and Cramer's results resolve themselves into a perfectly straightforward problem, involving the action of a bivalent salt in antagonising the destructive action of a monovalent one, on the normal stability of the bacterial cell. It is interesting to note, that they have been able to demonstrate the stabilising action of another bivalent salt, Sr; as this salt in the form of  $\text{SrCl}_2$  has about the same effect on colloidal aggregation as  $\text{CaCl}_2$ , this is what might be expected.  $\text{MgCl}_2$  on the other hand has little action in this respect, and they find it has no rupturing action. In regard to the action of  $\text{CaCl}_2$  on  $\text{MgCl}_2$ , this has also been pointed out by several other observers. Loeb (1906) for instance has called attention to the action of  $\text{MgCl}_2$  in modifying the action of  $\text{CaCl}_2$  on the muscular contraction of the disc of the medusa *Polyorchis*, and has pointed out that this is probably not one of real antagonism. The action of Na citrate in destroying the rupturing action of  $\text{CaCl}_2$  on the tissues is probably a purely chemical reaction in which Ca citrate is formed instead of Na citrate, NaCl also being formed.

An experiment was devised (Table XIII) to determine if Na citrate on being added to  $\text{CaCl}_2$  would prevent this salt from antagonising the action of the NaCl. A solution of three parts NaCl and one part Na citrate was mixed, and to this a trace of 0.125 M.  $\text{CaCl}_2$  was added. The solution was then adjusted by the addition of a little distilled water to have the same conductivity as Ringer's solution. A thick emulsion of *B. coli* was then washed in this solution several times and finally allowed to stand in it for an hour. It will be seen on consulting the following table, giving the details of this experiment, that at the end of this period the resistance of this emulsion had dropped very appreciably. It is obvious that the  $\text{CaCl}_2$  has failed to prevent the NaCl from lowering the resistance of the emulsion. Bullock and Cramer when they use these salts together are undoubtedly repeating the conditions of this experiment.

Table XIII.

Experiment to show the action of Na citrate on  $\text{CaCl}_2$ .

Solutions made up as follows:

3 parts NaCl, 0.124 M. of the same conductivity as Ringer.

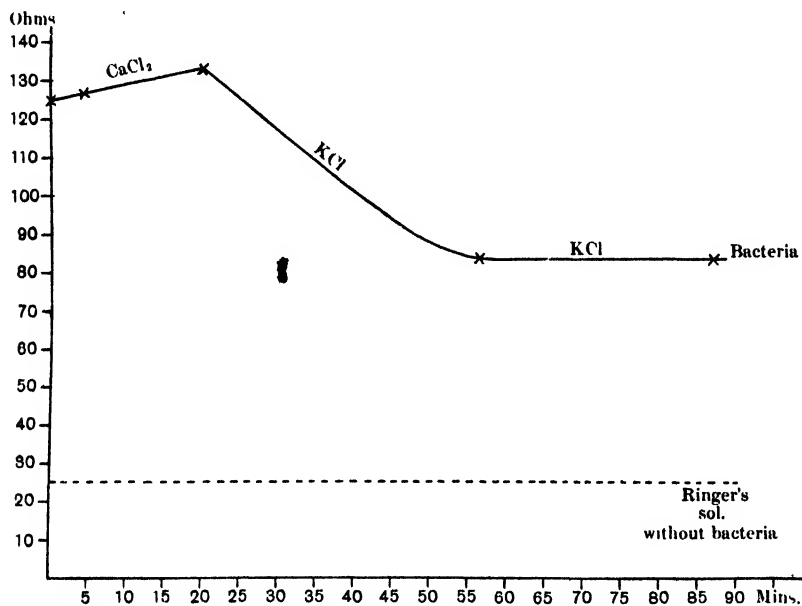
1 part Na citrate of the same conductivity as Ringer.

To 100 c.c. of above solution 1 c.c. of 0.125 M.  $\text{CaCl}_2$  was added and shaken. Distilled water was then added till the conductivity was the same as that of Ringer's solution.

1. Resistance of Ringer's sol. ... ..	85 ohms.
2. Resistance of NaCl plus Na citrate plus $\text{CaCl}_2$ ... ..	85
3. Resistance of <i>B. coli</i> emulsion in Ringer ... ..	131
4. Resistance due to bacteria, 131 - 85 ... ..	46
5. Resistance in NaCl plus Na citrate plus $\text{CaCl}_2$ after 1 hr ... ..	90.6
6. Drop in resistance in above solution after 1 hr. ... ..	40.6

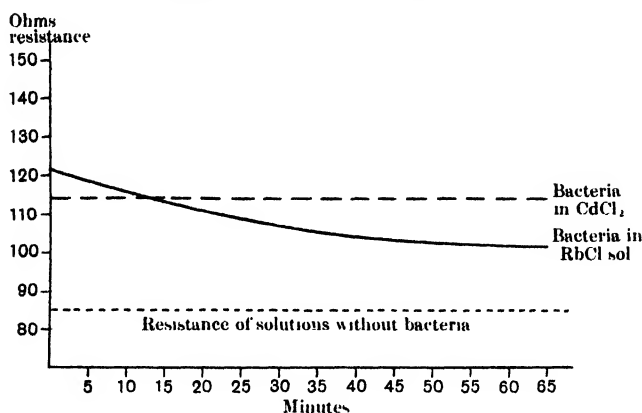
The  $\text{CaCl}_2$  in the above solution has not antagonised the action of the NaCl on account of the presence of the Na citrate.

Curve VII. Calcium chloride and Potassium chloride Experiment.



In a previous paper Cramer (1918) makes some observations on the action of NaCl and  $\text{CaCl}_2$  in affecting the growth of cancer cells. He found that if equal volumes (about 12 c.c.) of sterile M/7.5 solutions of NaCl and  $\text{CaCl}_2$  in tap water were placed separately in two test-tubes, and 0.5 c.c. of a fresh emulsion of cancer cells were added to each and shaken up for an hour or so, the emulsion in  $\text{CaCl}_2$  on injection into mice failed to grow as well as the control emulsions. The NaCl on the other hand grew at about the same rate as the controls, and the treatment with this salt had no effect in retarding the growth of the cancer cells.

Curve VIII. Rubidium chloride and Cadmium chloride Experiment. Temp. 25° C.  
Cell constant =  $9.5 \times 10^{-1}$ . Emulsion of *B. coli*.



Cramer does not state if the cancer cell emulsions were first washed several times in distilled water or several changes of NaCl before being finally allowed to remain in the NaCl, to remove all possibility of any Ca being brought over with the emulsion. It is well known that some cancerous growths may contain very considerable quantities of Ca. This amount of Ca combined with that possibly present in the tap water employed might very seriously interfere with the action of the NaCl, especially as the volume of NaCl used was rather small.

All these conditions may have been duly guarded against in this work, but no information is given to this effect in the description of the experiments.

In this paper as well as in the previous one it does not seem to have been realised what a small quantity of CaCl<sub>2</sub> is required to antagonise the action of NaCl. The ratio of these salts for instance in van't Hoff's solution being 2.3 to 100, and in the Ringer's solution used in the previous experiments the strength of the CaCl<sub>2</sub> is only 0.00187 M. as already mentioned.

The ions of one salt are said to antagonise another, when both are simultaneously present in a solution, and each prevents the other from entering the cell or exerting its specific action on the stability of the cell-wall and cytoplasm. Cramer therefore, in placing an emulsion of cancer tissue in a solution of M/7.5 CaCl<sub>2</sub> for a certain time and at the end of this period decanting the cells and resuspending them in M/7.5 NaCl solution for some time, is not strictly speaking dealing with a condition of true antagonism<sup>1</sup>. It is more or less a chance that under these circumstances antagonism takes place.

It is a question, moreover, if cancer tissue can be broken up and emulsified sufficiently to allow the NaCl to act on the cells. I have pointed out in my paper on the action of NaCl on the meningococcus that it fails to kill this

<sup>1</sup> These solutions are not isosmotic as stated in this paper.

Table XIV.

Experiments with *B. anthracis* on mice, a fresh 24 hr. culture being employed. The bacilli, after their conductivity had been determined in thick emulsions, were diluted down in each case to a strength approximately equal to about one million germs to the cubic centimeter; in this dilution they were injected in varying doses. 0 = animal alive and well, + = animal dead, h = hrs., d = days. All salt solutions used for these experiments had been carefully sterilized.

*B. anthracis*<sup>1</sup>.

No. mouse	Weight mouse	Dose and character of solution injected			Result	Remarks
1	18 grams.	0.25 c.c. in 0.124 M. NaCl sol.			+ 24 h	Small animal
2	22.5 "	"	"	"	0 4 d	
3	30 "	"	"	"	0 "	
4	27 "	"	"	"	0 "	Slightly ill after 48 hrs.
5	25 "	0.5	"	"	0 "	
6	28 "	"	"	"	+ 3 d	No signs of illness till 3rd day
7	31 "	"	"	"	0 4 d	
1	26 "	0.25 c.c. in Ringer's sol.			+ 24 h	Some of these had been dead sometime when examined at end of 24 hrs.
2	20 "	"	"	"	+ "	
3	29 "	"	"	"	+ "	
4	32 "	0.5	"	"	+ "	
5	27 "	"	"	"	+ "	
1	20 "	0.25 c.c. in CaCl <sub>2</sub> solution			+ 24 h	
2	26.5 "	"	"	"	+ "	
3	21.5 "	"	"	"	+ "	
4	26 "	0.5	"	"	+ "	
1	28 "	0.5 c.c. CaCl <sub>2</sub> , without bacteria			0 24 h	No signs of illness
2	30 "	"	"	"	0 "	
1	19 "	0.5 c.c. NaCl + CaCl <sub>2</sub> solution*			+ 24 h	
2	29 "	"	"	"	+ 48 h	
3	27 "	"	"	"	+ 24 h	

\* Composed of 200 c.c. 0.85 % sterile NaCl + 0.04 c.c. M/l CaCl<sub>2</sub> and adjusted to have the same conductivity as Ringer's solution.

Table XV.

Experiments with pneumococcus on mice. The pneumococcus strain had been passed through six mice previous to its use in this experiment. All emulsions were diluted down to approximately the same strength before being injected into the animals. 0 = animals alive and well, + = animal dead, h = hours, d = days.

*Pneumococcus.*

No. mouse	Weight mouse	Dose and character of solution injected			Result	Remarks
1	25 grams.	0.25 c.c. in Ringer's solution			+ 24 h	
2	30 "	"	"	"	+ "	
3	21 "	0.5	"	"	+ "	
4	23 "	"	"	"	+ 48 h	
1	31 "	0.25 c.c. in 0.124 M. NaCl sol.			0 24 h	Dead 3rd day
2	19 "	"	"	"	0 "	
3	25 "	"	"	"	0 "	
4	22 "	"	"	"	0 "	
5	28 "	0.5	"	"	0 "	Dead 8th day

<sup>1</sup> The groups of animals to which the three consecutive tables relate were in each case injected with the same bacterial emulsion.

Table XVI.

*B. anthracis.*

No. mouse	Weight mouse	Dose and character of solution injected	Result
1	26.5 grams.	0.25 c.c. Ringer's solution	+ 24 h
2	31 "	" " "	+ "
3	29 "	" " "	+ 48 h
1	22 "	0.25 c.c. in 0.124 M. NaCl sol.	0 3 d
2	27 "	" " "	0 "
3	29 "	" " "	0 "
1	30 "	0.25 c.c. in 0.124 M. NaCl + trace Ca nitrate	+ 24 h
2	25 "	0.25 c.c. in 0.124 M. NaCl + trace Ca nitrate	+ "

germ if any clumping of the cocci takes place. On subsequent incubation, the bacteria in these clumps grow and give rise to isolated colonies; the NaCl seem unable to affect the cocci in the interior of the clumps. In order that the NaCl may kill all the bacteria, it is necessary to employ emulsions entirely free from clumps or masses of bacteria. I fail to see how cancer tissue can be emulsified so thoroughly that all the cells are separated, and the NaCl given free scope to act.

I believe this reason, as well as the fact that no special precautions were taken to see that no Ca was introduced with the cancer tissue cells in these experiments, accounts for the failure to get the action of the NaCl. In these experiments the real action of the NaCl on the cancer cells has not been properly demonstrated. There is reason to think that if they were repeated with due attention to these points, the action of this salt on the cancer cell would be the same as on all other animal and plant cells. That sufficiently long exposure to pure solutions of NaCl in a concentration of 0.145 M., say two or three hours, would result in the death of these cells.

Thus the failure to distinguish the characteristic action of NaCl on living cells, and its highly reversible nature, renders the results of Cramer, Bullock and Cramer open to a different interpretation from that given by them.

No evidence has been advanced by Bullock and Cramer to show that the action of the NaCl on the bacteria is not the decisive factor in the matter. If a similar effect could be produced by the injection of the bacteria in distilled water, there might be some ground for believing that the action of the Ca was on the tissues. If on the other hand it can only be obtained by the injection of bacterial suspensions that have been washed several times in NaCl, in the light of the present conductivity experiments the explanation is obvious.

We come finally to the question as to what do these changes in the conductivity of bacterial emulsions signify? Osterhout, in his experiments on plant tissues, believes that they give us definite values for the permeability of the tissues to the ions. This view has been openly challenged by Stiles and

Jørgensen (1918), who point out that this is to a certain extent a pure assumption.

In placing living cells in pure salt solutions, no matter how weak, it is doubtful if we are investigating permeability at all, but simply the destructive action of the salt on the cell, as the result of which a certain amount of ex-osmosis or endosmosis takes place. Neither Osterhout's experiments, nor those of Stiles and Jørgensen, or Stiles and Kidd (1919), give us any information as to how absorption takes place from a balanced solution. It would seem that the problem is one more for the protein chemist than for the experimental physiologist.

Sørensen (1917), in his extensive studies on the physical properties of proteins, has shown that the capacity of egg-albumen to combine with acids and bases, at a certain hydrogen ion concentration, is a function of the amount of ammonium sulphate present, and is greater as the latter increases.

It is possible on a basis of a measurement of the number of hydrogen ions present in a solution of egg-albumen, having a known composition and containing ammonium sulphate, to estimate the total content of surplus acid, and to determine by formula its approximate distribution between the two phases of the egg-albumen solution.

It will perhaps be from investigations of a similar character, on the action of the ions of Na, K, and Ca, on the physical properties of the proteins and lipoids, that will show us how these ions stabilise the cell membrane and cytoplasm, for it is clear that in the presence of these ions the semi-permeability of the cell-wall remains constant.

Schryver (1913), in a series of researches on the formation of gels from cholate solutions, has shown that Na cholate solutions set to a gel when heated in the presence of Ca. This gel formation is readily inhibited by the presence of relatively small quantities of NaCl. In further experiments, he showed that cholate gels are eroded when immersed in solutions of NaCl and other chlorides, and that this erosive action can be antagonised by very small amounts of  $\text{CaCl}_2$ . Quantitatively, the chloride solutions in their erosive action differ considerably from one another, the order of their action being as follows commencing with the greatest, LiCl, NaCl,  $\text{MgCl}_2$  and  $\text{KCl}^1$ . It will be seen that we are dealing in these experiments with similar conditions to those demonstrated by the previous conductivity experiments on living bacteria.

In order to see if the solutions found by Schryver to produce erosion of a cholate gel would also produce a change in the conductivity of bacteria emulsions, certain experiments were made; they all gave negative results, however, where chloroform and chloral hydrate<sup>2</sup> were used, two substances that gave the highest cholate gel-destroying capacity in Schryver's work.

<sup>1</sup> The effects of the action of these salts on the permeability of vegetable tissues follows the same order, as determined by Stiles and Jørgensen (1915). In the case of bacteria no attempt was made to determine the order of their action, as the resistances employed were too low to allow of any relative comparison.

<sup>2</sup> These experiments need repetition.

## IV. SUMMARY.

The action of univalent and bivalent salts on bacteria in affecting the conductivity of thick emulsions of the meningococcus and *B. coli* demonstrates the important fact, that they alter the conductivity of these germs in the living condition, in a very definite manner. In this alteration, the predominant part is played by the cat-ion.

All monovalent cat-ions, with the exception of the H-ion, such as those of Na, K, Li, Rb, produce a rapid increase in conductivity or a fall in resistance. In its early stages this increase in conductivity is readily reversible in these solutions. If allowed to follow its due course, however, it leads finally to death in about two hours, when the bacterial cells no longer offer any resistance to the passage of the ions. The conductivity of the emulsion then becomes that of the fluid in which the emulsion has been suspended.

Bivalent cat-ions and the H-ion, on the other hand, at first produce a slight fall in conductivity or an increase in resistance, followed, secondly, by an irreversible increase in conductivity which is slow and gradual resulting finally in death after 48 or more hours. This is shown by the cat-ions of Ca, Sr, Ba, and Cd.

In a balanced solution such as sea-water, blood plasma, Ringer's solution, van't Hoff's solution, where a certain quantity of  $\text{CaCl}_2$  antagonises a larger amount of NaCl and KCl, the conductivity of bacterial emulsions undergoes no change, but remains constant.

Dead in distinction to living bacteria offer little resistance to the passage of ions of a solution. It would seem the relatively high resistance of the bacterial cell is due to some condition present in the living and absent in the dead state.

It has been shown by experiments on animals that the condition of increased conductivity of the cell is coupled with a loss of virulence in the case of some pathogenic bacteria. It is not clear from these experiments if this is due to the actual death of the germs in a monovalent salt solution, or to a detoxicating action of these solutions on the germs. It would seem there is some evidence for believing that the latter takes place, as the lost lethal power returns in the presence of a small trace of Ca. In bivalent salt solutions no loss of toxicity takes place. It has been pointed out that the action of monovalent and bivalent salts on bacteria offers a new explanation of certain experiments with gas gangrene organisms, where it is at present considered that the action of the salts are on the tissues of the animal and not on the bacteria.



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## THE CULTIVATION OF AEROBIC BACTERIA FROM SINGLE CELLS.

By EDWARD C. HORT, F.R.C.P. (EDIN.).

(With 1 Text-figure.)

IN order to obtain cultures of aerobic bacteria from single cells it is hardly necessary to insist that a method of isolation should be employed which can be relied on to ensure that one organism, and one only, is the starting point for inoculation of the first subculture. Unfortunately, for technical reasons which will be explained, no such method is at present available.

The object of the present note is to show why the present methods of isolation are unsatisfactory, and at the same time to describe a simple and convenient method on which reliance can be placed. The methods at present in use include the fragmented glass method, the Indian ink method, the squared coverslip method, the capillary tube method, the Barber method, and the droplet method, of which the Malone method is a modification. It is assumed that the principles underlying the use of these methods are sufficiently well known to preclude the necessity for a detailed description of each. The fallacies and drawbacks incident to their employment will therefore mainly be dealt with, though a certain amount of description of the less well-known methods is unavoidable.

### THE FRAGMENTED GLASS METHOD.

By this method of isolation, a description of which I am unable to find, an attempt is made to superimpose on selected single cells, scattered over agar or gelatin, small fragments of thin glass in the hope that successful inoculation of a suitable medium, such as broth, will result by placing a selected fragment with its adherent organism in such medium. The disadvantages of the method are as follows:

1. Only dry lenses can be employed.
2. The poor visibility of organisms lying under the prismatic edges of the fragments of glass often makes it impossible to be certain that only one organism is present under the fragment of glass selected.
3. When picking up the selected fragment it is difficult to be certain that organisms lying close to the fragment are not also unwittingly picked up by slight displacement of the fragment during the process of removal.

4. Once the selected fragment has been picked up it is difficult, or impossible, satisfactorily to re-examine it with a view to determining the presence or absence of organisms not previously noticed.

5. As usually employed no control observations of growth from single cell to single colony can be carried out to determine the presence or absence of more than one organism, because the selected fragment is at once placed in broth or other suitable liquid medium. It is of course possible to select a fragment of glass which appears to be lying on one organism only, to incubate the culture until colony formation is well established, and to examine again before removal. In practice, however, disadvantages 2 and 3 come into operation, and invalidate this method of control. Apart from disadvantages 1 to 5, which together prohibit the use of the fragmented glass method for scientific work, there are two minor drawbacks to the method. The first of these is the mechanical difficulty of superimposing small fragments of glass over selected organisms, there being often about 50 per cent. of failures even after considerable practice. And the second lies in the fact that frequently removal of the selected fragment fails to remove also the organism desired.

#### THE SQUARED COVERSIP METHOD.

By this method, described by Hewlett, a large coverslip is employed on which are etched several numbered squares. On the coverslip is poured a thin layer of agar, or gelatin-agar, a minute quantity of a liquid culture of the desired dilution being spread with a glass rod over the agar when set. The coverslip is then inverted over a hollow moist cell, and examined with a dry lens. The position of an individual organism in a given square is then noted, and the culture is incubated. When colony formation is sufficiently marked to be visible to the naked eye the selected colony is removed, and dealt with in the ordinary way. In theory this method sounds promising, because it would appear to allow of control observations being made during development from single cell to single colony, and so of ensuring that one organism, and one only, is the starting point of the desired culture. In practice, however, the method is of little or no value for the isolation of bacteria, especially when small, because the visibility of organisms on an inverted culture is extremely poor, even under optimum conditions of illumination. It is therefore impossible to be certain that only one organism is present in a given square, and that a colony which appears to be derived from one cell only is not in reality derived from two or more coalescing colonies from two or more adjacent cells. And the necessity for waiting until colony formation is sufficiently far advanced to allow of removal under the naked eye greatly increases this danger.

## THE INDIAN INK METHOD OF BURRI.

For accurate work this method, described in detail by Besson, is not satisfactory, owing to the tendency to concealment of minute organisms by the pigment, although theoretically they should stand out in bold relief even when near to the vanishing point of vision. Numerous experiments with Congo-red, and other pigments, have convinced me that these suffer from the same defects as does Indian Ink.

## THE CAPILLARY TUBE METHOD.

In 1916 I experimented for several weeks with round, and with flat, capillary glass tubes filled with dilute broth or gelatin cultures. The best results were obtained with extremely fine flexible strands of tubular glass, attached in short lengths to microscope slides with plasticine, the desired section containing the selected organism being excised with a sterile knife, and dropped into broth. Prolonged examination of these sections at a temperature which precluded multiplication showed however that even under optimum conditions of illumination the method is a treacherous one, and it was therefore abandoned for the isolation of aerobic organisms, though the fact that it is possible to examine these fine tubular threads of glass with an oil-immersion lens was a strong temptation to continue the experiments. A further drawback to the method for aerobic organisms, unless freely motile, is the difficulty of ensuring passage of the contained organism into the surrounding broth, multiplication within the tube apparently not taking place. For anaerobic organisms, as recorded in 1918 by Holker, the method has obvious advantages, though the optical difficulties of ensuring the presence of single organisms still remain.

## BARBER'S METHOD.

By this method an attempt is made to isolate single cells from liquid cultures with the aid of minute pipettes held in a mechanical finger operated by an ingenious adjustment device. This method is unreliable because

(a) Once the pipette has been removed re-examination to determine whether more than one organism is or is not present is impossible.

(b) Control observations of development from single cell to single colony cannot be carried out owing to the exclusive use of a liquid medium in the early stages.

(c) Of the optical difficulties attendant on the examination of droplets (*vide* "the droplet method").

## THE DROPLET METHOD.

In theory this method is a good one, because it allows of the use of an oil-immersion lens. It may be used in one of three ways:

1. A sharp pointed spud of hard wood, sterilized by immersion in 40 per cent. formalin, and dried just before use in sterile wool, is dipped

into a broth culture of the desired dilution. Momentary contact is then made between the point of the spud and the centre of a minute ring cut on a sterile coverslip with a revolving diamond. The inoculated coverslip is then inverted onto the upper edge (moistened with Canada balsam) of a glass collar cemented to a microscope slide, and is examined with a dry lens. The minute droplet is easily recognized in the centre of the etched ring, and provided that its diameter is not greater than that of the microscopic field it can be thoroughly examined in a few seconds with the dry lens, and in a few minutes with an oil-immersion lens. A series of rings, each with its own droplet, can be examined in turn on each coverslip till one droplet is found, apparently containing only one organism. The coverslip is then removed and placed on its back in a sterile Petri dish. A small drop of broth is then delivered into the centre of the ring containing the selected organism, and is recovered with a fine sterile pipette, delivery being finally made into a tube of broth.

2. Delivery is effected by depositing minute droplets with a hair pipette attached with plasticine to a pipette of larger bore provided with a rubber teat, accurate delivery being effected by slight compression of the rubber teat at the exact moment of contact of the tip of the pipette with the centre of each etched ring. As in droplet method I, to ensure rapid examination, the diameter of the drop should not exceed that of the microscopic field afforded by the dry lens employed in the preliminary search.

3. Delivery is effected, on an inverted coverslip in a closed glass cell, of a series of minute droplets from a fine pipette by an ingenious arrangement devised by Malone, using two microscope stands for the purpose. Each droplet is examined in turn, and the selected droplet is finally collected with a fresh pipette, using the same mechanical device for collection as for distribution.

In practice the droplet method, whether delivery be effected by wooden spuds, by the direct pipette, or by the inverted pipette of Malone, is disappointing. This is because the minute size of the drop which must be employed to avoid grave error often involves a fatal retraction of the periphery of the drop, with the danger of leaving stranded in the shrinkage area—even in a moist cell—organisms which rapidly fade from view, but which nevertheless, as experiment shows, are not necessarily dead. This retraction difficulty can be to some extent avoided by using a 20 per cent. solution of glycerin in water as the bacterial vehicle, though the percentage of successful cultivations is in practice small owing to the lethal effect of the necessarily high concentration of glycerin. Even apart however from the retraction difficulty the visibility of bacteria in minute hanging droplets is always poor, and makes it impossible to be absolutely certain that one organism, and one only, is present in the droplet it is desired to use as inoculum. And finally, as in the

case of other methods involving the use of liquid media throughout, it is not possible with any modification of the droplet method to carry out control observations of development from single cell to single colony.

We learn, then, from this brief review of the methods of isolation at present available that for the reason just stated

1. Control observations from single cell to single colony are absolutely essential because of the optical difficulties which attend all known methods, making it impossible to rely upon any results from single observations.

2. Any method which aims at isolation from liquid media is inadmissible. This at once excludes Barber's method, all the droplet methods and the capillary tube methods.

3. For the same reason the fragmented glass method and the Indian Ink method must also be rejected.

We are thus left with only the squared coverslip method which has greater optical disadvantages than any other method, disadvantages which are inseparable from the use of an inverted medium inoculated on its distal surface.

It would seem therefore that the only chance of finding a reliable method of isolation is to employ direct examination of organisms on a solid medium, and to insist on a series of control observations in order to be certain that in the development from cell to colony one cell, and one cell only, is originally present.

At a time when it was believed that bacilli or cocci can only arise from pre-existent bacilli or cocci by equal binary fission the necessity, especially in the case of the larger organisms, for control observations during development from single cell to single colony was—naturally enough—not apparent. Now, however, that it is known that, for example, minute gonidial forms extruded from the mother-cell are often themselves highly fertile it is clear that complete control observations are essential in order to ensure that the unsuspected presence of these minute forms does not give rise to serious error.

Of the two methods now to be described one is suitable for oil-immersion work, and the second for dry lens work only.

#### FOR OIL-IMMERSION LENS.

A series of sterile coverslips is prepared, each with a small ring etched on one of its surfaces. At the same time is also prepared a dozen or more clean sterile microscope slides, over each of which is poured under cover of a Petri dish filtered peptone-agar in a thin layer. A dilute culture is now prepared, and in turn each coverslip is inoculated in the centre of the etched ring with the minutest possible droplet of the culture. Each inoculated coverslip is now placed face downwards on each agar slide, care being taken to ensure direct application of slip to agar without sliding of the former over the latter.

Great care must also be taken to ensure that the droplet of inoculum is sufficiently small not to run outside the ring when firm pressure is exerted over the slip when *in situ*. With a little practice this accident, which is of course fatal to the experiment, can be avoided, it being quite possible to effect delivery of a droplet so small in diameter as to be hardly visible to the naked eye, and well within the field of vision allowed by a dry lens of  $\frac{1}{8}$ -in., and the appropriate ocular. After careful search with the dry lens an oil-immersion lens is substituted, and the whole area within the etched circle is thoroughly examined. If only one organism can be found the immersion oil is carefully removed, and the slide is incubated at 37° C. for four to six hours, being examined at intervals of 20 minutes throughout this period.

When the observer is perfectly satisfied that the colony now in process of formation has started from the original cell, careful drawings being made throughout, and that only that particular cell was originally present within the etched ring, the slide is now replaced in the incubator. Twelve to eighteen hours after inoculation the coverslip is removed with sterile forceps, and a platinum loop charged with broth is rubbed over the area of glass enclosed by the etched ring, the second subculture being then carried out in the ordinary way.

This method, using an oil-immersion lens, is tedious on account of the necessity for removing the oil used at each examination, preparatory to each re-incubation. The brilliancy of outline produced by pressure of the coverslip on the agar surface and the increased amount of detail to be made out by using an oil-immersion lens, more than compensate however for this trifling drawback. And if it is desired, as was the case in most of my studies, to obtain careful drawings of warm-stage development of morphological changes in the passage from single cell to single colony it is incomparably the best method, though the relative loss of oxygen necessitated is certainly a drawback.

The accompanying figure illustrates what actually happens in such a case, and demonstrates how clearly the morphological changes can be followed by using this method, though in this particular case the experiment was not undertaken with a view to subsequent identification of the nascent colony shown.

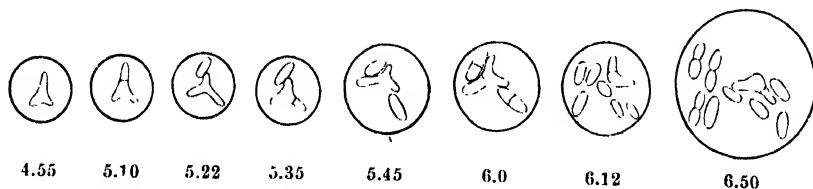


Fig. 1. *B. typhosus*. Record of development of a colony observed between 4.55 and 6.50 p.m. on + 10 agar, from 4 % glucose broth.

## THE PERFORATED PLATE METHOD—FOR DRY LENS ONLY.

For ordinary purposes of isolation of single cells, however, in which all that is required is a series of careful control observations to ensure that a culture has been started from one organism only—without any necessity, that is, for elaborate studies of morphological changes in individual cells as illustrated in the figure—the use of the dry lens alone is sufficient. An ordinary sterile glass slide is covered with a suitable medium, such as filtered peptone-agar or glucose peptone-agar, by pouring the latter at a temperature of as near 100° C. as possible under cover of the top of a sterile Petri dish. A liquid culture in appropriate dilution is then spread over the medium, as soon as set, with a glass rod, and a thin strip of perforated celluloid, or other suitable material, is placed over the slide. Sterile coverslips are now placed lightly over the celluloid, and the slide is placed within a sterile Petri dish, and is incubated at 28° C. for from 1 to 1½ hours. The object of placing coverslips over the celluloid is firstly to protect the culture medium from dust during subsequent examinations, and, secondly, to convert each of the tiny cells into a moist chamber, aqueous vapour arising from the surface of the moist medium becoming recondensed on the inverted coverslip.

At the end of 1 to 1½ hours the slide is examined cell by cell until the organism it is required to isolate is found, alone in a single cell, or until a cell is found in which a small number of suitable organisms occur so conveniently placed that there is no danger of the resulting colonies impinging on each other. When found the position of the selected cell is noted by the vernier attached to the microscope stage, and is recorded on a diagram with drawings of the organism or organisms it is desired to isolate. The slide is now replaced in its Petri dish and reincubated. It is further examined at short intervals until colony formation from single cells is established, careful search on each examination being made to ensure that organisms previously unrecognizable as bacteria are not now coming into view, and to make, if desired, such careful drawings of alterations in morphology of the organisms originally selected, or of their direct descendants, as is possible with a dry lens. When the colonies have reached a convenient size it will be found that they are still too small for removal under the naked eye, and if they are left until recognizable without the aid of a lens there is always the danger of coalescence of adjacent colonies—if a cell has been selected with more than one organism—and of the experiment being ruined.

If, however, the following simple technique is adopted there is no difficulty in picking off fragments of colonies—the growth of which from individual cells has been watched and recorded *de initio*—whilst yet too small to be detected under the naked eye. The procedure is as follows. The objective in use is replaced by a perforated metal stop, made for the purpose by Angus and Co., Wigmore Street, London, the perforation being conical in shape, with the apex at its lowest point. Into this conical space is dropped a No. 9 solid



steel needle, the point of which, when *in situ*, is blackened in the flame of a match. A minute drop of paraffin wax on a slide has, previously to removal of the objective, been exactly centred on the microscope stage. The blackened needle is now lowered till it touches the wax, the needle receding in its holder at the moment of contact. The position of the blackened point of contact on the white wax is now noted under the replaced objective, and is brought into the centre of the field, if not already there. The whole manoeuvre is repeated a second time in order to be sure that the point of contact is sufficiently approximate. The selected cell, from which the coverslip has been removed, is now examined, and the chosen colony is centred on a hooded stage. The point of the resterilized needle is now touched with broth in a small platinum loop, is brought into contact with the centre of the colony and, after racking up, is again touched with broth in a resterilized platinum loop, from which a tube of broth is inoculated in the ordinary way. Finally the objective is once more restored, and a control observation made that the desired colony has been touched, and no other.

The advantages of the perforated plate method are the following:

1. Extreme simplicity.
2. The expense of the outfit is negligible.
3. It is relatively rapid.
4. The method of cultivation ensures the maximum of oxygen and moisture.
5. Successful cultivation from fragments of the removed colonies takes place in my hands in 100 per cent. of the cases.
6. It affords an excellent control observation throughout against
  - (a) contamination,
  - (b) picking up fragments of a colony from more than one organism.

In conclusion it is necessary to point out that cultivation of bacteria from single cells is, even when employing a good method, a most tedious procedure, involving several hours' close work for each organism isolated, if the results are to be relied on. It cannot be too strongly insisted that all claims to have grown cultures with certainty from single cells must be accepted with reserve unless the whole process has been repeatedly controlled from selection of single cell to transference of the established colony, either in whole or in part.

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## THE REPRODUCTION OF AEROBIC BACTERIA.

By EDWARD C. HORT, F.R.C.P. (EDIN.).

(With Plates IV—VII.)

### PART I.

UNTIL the year 1916 it was generally believed that the non-sporing bacteria of the lower orders are only capable of reproducing themselves by the simple process of transverse binary fission into two equal parts. This—it was taught—is the beginning and end of their reproductive life. A bacillus, or a coccus, always arises directly from an equally dividing bacillus or coccus, and in no other way whatever. So firmly fixed was this conception of the entire reproductive life of the lower bacteria under all circumstances that any evidence suggestive of the occurrence of other methods of reproduction, such as by budding, or by branching, or by the production of endobodies was apt to be explained away either by alleged contamination of the cultures employed, or by vague references to the phenomena of involution.

In 1916 and 1917, however, and again in 1918, I showed by several series of warm-stage studies of isolated organisms of the enteric group that under certain circumstances the lower bacteria are able to reproduce themselves by the production of fertile branches and buds, and by the endogenous production of gonidial bodies, in addition to the more familiar method by equal binary fission. And I also showed that evidence of this can be found, if looked for, in young cultures in standard media. The cultures I employed were, as I thus proved, pure cultures, so that any suggestion of contamination organisms being present to account for the results obtained was out of the question. And as I had shown that organisms undergoing branching or budding were often fertile organisms, the detached buds and branches themselves giving rise to a new race, it was no longer possible to advance the theory that these were involution forms—in the ordinary sense of the term—since organisms undergoing genuine involutionary changes, recognizable as such, are sterile organisms. The thesis that the lower bacteria can reproduce themselves in more ways than one, when occasion arises, was therefore fully established for the first time, there being no previous record of continuous warm-stage observations to show that isolated organisms belonging to the lower orders of bacteria, and undergoing branching or budding, or endogenous chromatinolysis are capable of perpetuating a new race which is itself fertile, its progeny being culturally, biochemically and serologically identical with the original mother-cell.

Of the experimental observations establishing these facts several were published in 1916 and in 1917. They therefore need not be reproduced here. Of the remaining observations, and the most complete, a few were examined by a War Office Committee (*v. note infra*) specially appointed for the purpose, and are now published—together with further observations—for the first time.

#### INVOLUTION FORMS: GENUINE AND SPURIOUS.

Before presenting the chief facts on which the above statements are based it is necessary to attempt a definition of the vague term involution form, and to show, in passing, how the literature of bacterial morphology is pervaded with misconceptions arising from the lack of any clear understanding of what an involution form, properly so-called, really means.

An involution form of bacterium can only mean a bacterium which is undergoing retrogressive, or perhaps, degenerative changes. It is, strictly speaking, a sterile organism which is not only incapable of maintaining its reproductive activity, but is also incapable of maintaining its integrity of form. It stains irregularly, feebly or not at all: and in the unstained condition it rapidly fades from view as death ensues, and as autolysis proceeds. It becomes misshapen and deformed, quite early—in the case of motile organisms—losing its motility, as well as that perfect symmetry of outline, and diffused receptivity of stain which mark the healthy normal organism. To the trained observer it offers no difficulty of recognition, and is clearly an organism that has failed in the race of life, as a result of profound disturbance—set up by prolonged sojourn in unfavourable environment—of that power of adjustment of osmotic variations on which all cell activities ultimately depend.

There are, however, often to be seen under less unfavourable conditions aberrant types of the lower bacteria, aberrant that is in the possession of buds or branches, or in some other way, which show none of the characteristics of the genuinely involuting organism. They exhibit perfect symmetry of form, they show no irregularity of staining, which is often deep—especially with carbol-fuchsin—and they do not fade from view in the unstained condition. These organisms are none the less often indiscriminately ranged with genuine involution forms, and are often figured as such, when they do not escape detection altogether, in some of the text-books. So general indeed is the tendency to apply the term involution to any form of bacterium departing from the usual that the legend is unblushingly transcribed from book to book. And the result has been that until recently no effort has been made to apply the crucial test of isolating healthy looking bacteria of the lower orders undergoing branching or budding, and of then determining their ability or inability to produce a new race culturally, biochemically and serologically identical with normal organisms. If an aberrant type of bacterium—neither bacillary nor coccoidal—can be shown to be endowed with fertility it is clearly not an involution form in any reasonable sense of that ill-used word. And yet till

1916 there were no adequate observations on record that this, the supreme test of bacteriological science, had been applied to the lower bacteria to determine whether the current belief in their ability to divide by equal binary fission only was, or was not, well founded: though in the case of other organisms, the existence of higher orders of which appeared thus to be established, the test had already been successfully applied. No effort, in short, was made by applying scientific methods of precision to separate false involution forms from genuine.

For example within the past 25 years several papers have appeared dealing with aberrant morphological types of the lower bacteria, some of the authors, such as Lehmann and Neumann in 1896, on insufficient evidence regarding the occurrence of branching and of budding as a phase of normal development. Others, such as A. Fischer in 1897, and Migula in 1900, regarded the occurrence, again without offering satisfactory evidence, as a pathological process induced by cultivation in unsuitable media. Others again record their observations without comment, or without offering any evidence allowing of interpretation of the results shown. In most of the recorded observations the one constant factor in the media employed for demonstrating morphological aberrancies, indiscriminately classed as involution forms, has apparently been the presence of free H ions, both from organic salts and from inorganic, or the addition of relatively non-toxic doses of the various aniline dyes, perhaps acting in the same direction. Thus Vedder and Duval in 1901 noted that in cultures of dysentery bacilli on glucose agar aberrant types were sometimes found which, without further investigation, they thought were exclusively involutionary in character. In 1900 Fischer recorded that he had placed the *V. cholerae*, the *B. anthracis*, and other organisms in hypotonic solutions of salt in water containing glycerin, and he described a condition to which he gave the term plasmoptysis. Spherical swellings appeared, anywhere in the bacillary axis, filled with plasma extruded from the mother-cell, the plasma pushing the cell-wall, at a weakened spot, he said, in front of it. Some of these spherical swellings gradually expanded and faded from view after varying periods of immersion. In these extreme cases there can be little doubt that the process was the result of a lethal disturbance of osmotic equilibrium set up by prolonged immersion in a fluid with a lower concentration of solutes outside the cell than within it. But Fischer produced no evidence that organisms undergoing lesser degrees of plasmoptysis, in virtue of immersion for shorter periods of time, had lost their reproductive powers. And he produced no evidence to show whether the new spherical bodies produced by plasmoptysis were themselves fertile or sterile. And he therefore provisionally concluded that all degrees of plasmoptysis are necessarily involutionary in character, although he admits that he had not tested the point. To this view also Abbott and Gildersleeve, writing in 1904, in an essay marked by sound critical acumen, were also inclined in their explanation of the occurrence of branching and budding forms in their acid cultures of the *B. diphtheriae*:

though they too were careful to state that they had been unable to determine whether their buds and branches were or were not capable of producing a new race, a criterion to which they evidently attached great importance. In 1904 Ainley Walker and Murray noted the occurrence of branching forms of the *B. typhosus* in media containing gentian violet, fuchsin-methyl green, or methylene blue, and in 1912 Revis dealt with the cultivation of coliform organisms in media containing malachite green. In addition to these there are numerous other recorded observations of morphological aberrations, notably those by Almquist, by Sopp, and by Norsk, to which access has so far not been possible, by Horrocks in 1911, and by Löhnis in 1916.

In all these papers however, with the possible exception of those not yet studied, there is, as stated, no single observation on record that any of these branching or budding forms had been isolated with a view to determining not only their own fertility, but also that of the buds and branches themselves, either before or after their separation from the mother-cell. In addition to all these observations on the occurrence of plasmoptysis phenomena it has not infrequently been noted that under certain conditions plasmolytic changes may be set up in bacteria, leading to endo-fragmentation of chromatin. In bacilli and in cocci so affected, and the change may also be demonstrated in streptococci and streptobacilli, minute dots of deeply-staining material make their appearance. This can often be readily made out in unstained living preparations, which may also contain these bodies in large numbers after extrusion. Photographs of drawings of these bodies were shown by me to the Royal Society in 1916. This endo-fragmentation, described by Fischer in connection with his studies on bacteriolytic serums, has again generally been looked upon as a purely involutionary phenomenon, there being again no recorded warm-stage observations to determine the sterility or fertility of these minute bodies after extrusion from the mother-cell.

#### PHYSIOLOGICAL AND PATHOLOGICAL DEGREES OF PLASMOPTYSIS AND PLASMOLYSIS.

From what has been said it is clearly a matter of fundamental importance to determine whether the occurrence of plasmoptysis in bacteria is a pathological process only, or whether it is, if disturbance of osmotic control be not too profound, a genuine physiological process. Demonstration of unfamiliar methods of reproduction of organisms undergoing moderate degrees of plasmoptysis, combined with demonstration of the fertility of the new race, would unquestionably prove that the phenomenon does represent a phase in physiological development when environmental conditions become difficult, and, incidentally, that bacteria can reproduce themselves in more ways than one. The problem as to the exact point at which physiological processes end, and pathological processes begin, may safely be left to the curious in such matters. It also appears to be a matter of equal importance to determine whether the occurrence in bacteria of the opposite phenomenon of plasmolysis—induced

by osmotic disturbances set up by a lower concentration of solutes within the cell than outside it—is a pathological process only: or whether it too, when kept within proper limits, does not represent a phase in physiological development.

In the case of plasmoptysis where the crucial point in distinguishing between physiological and pathological events appears to be the potential fertility of the extruded plasma in its new cell-wall, so in the case of plasmolysis the crucial point in giving the correct answer appears to lie in proving or disproving the potential fertility of fragmented chromatin within the cell itself, or outside it. In the former case, that of plasmoptysis, the extruded elements would appear to be the expression of exogenous methods of reproduction by unequal binary fission, giving rise to fertile branches, buds and spherical bodies. And in the latter case, that of plasmolysis, the fragments of intracellular chromatin would appear to be the expression of endogenous reproductive activity equivalent in effect to the exhibition of multiple gonidia: the fertility of the new bodies produced both by exogenous and by endogenous methods of reproduction being finally expressed in the ordinary vegetative forms familiar to laboratory students.

In the appended photographs of drawings will be seen some of the numerous types of organisms observed in 4 per cent. glucose agar, or 4 per cent. glucose broth, cultures from a single colony isolated from the faeces of a severe case of typhoid fever which proved fatal on about the fourteenth day of the disease. Indisputable evidence of the direct relationship of many of these forms to the *B. typhosus* is given below.

#### PLATE IV.

The drawings in Plate IV represent composite selected fields, that is to say, they do not represent fields as actually observed, each drawn field containing selected organisms from numerous fields, the basis for selection for each field being merely the type of organism it is desired to illustrate. Each field is designated with a separate label, with the suffix -oid, merely to show how closely many of the types depicted may simulate parasitic fungi, and to facilitate description.

For example:

Field	2	illustrates various types of bacteroid		
„	3	„	organisms	resembling sporangia
„	4	„	„	„ chlamydo-spores
„	5	„	„	„ oidia
„	6	„	„	„ gonidia
„	7	„	„	„ cocci

Fields 1, 2, 3, 4, 5, 9, 10 mainly represent unstained living organisms, drawn, with the exception of 3, by the aid of the camera lucida.

Fields 6, 7, 8 represent stained organisms drawn mostly by freehand.

The following types were found to be highly motile, this motility being repeatedly confirmed during the eight months that this strain was under daily subcultural observation:

Bacteroids	Oidioids
Sporangioids, attached and detached	Gonidioids.
Chlamydo-sporeids	

The only types in which motility was not observed were the thick-walled resting cells depicted in Field 1, and the thin-walled coccoids seen in Field 7. The motility of these there is no record of.

As noted in Plate IV, each field represents organisms observed in cultures on plus 10 agar varying in age between 1 and 4 hours, subcultured from 4 per cent. glucose broth cultures varying in age between 12 and 18 hours. In all cases the maximum incubator temperature employed was 28° C. to 30° C.

The following short description of each field will be sufficient to bring out the points it is desired to emphasize.

Field 8 is of an ordinary plus 10 broth 8 hour *B. typhosus* culture of a strain which had never been grown in media containing glucose. In addition to "normal" bacilli, and one or two organisms exhibiting Artaud's nodes, are to be seen three deeply-staining forms, two bacillary and one bacteroidal, such as are discussed in full in the text. In these deeply-staining forms, which—as here—may or may not be aberrant in form, lies the key to the problem presented by aberrancy of bacterial form. Hitherto these deeply-staining forms, often known as giant-forms, have been looked upon as involution forms, carrying the suspicion of being sterile forms. Actually they are highly fertile, and may exhibit one or more of many different types of reproductive activity. Possession of this deep receptivity of stain appears in fact to presage an explosion of reproductive activity. These organisms are readily made out in the living unstained condition, and can usually be relied on to demonstrate their fertility on the warm-stage when environmental conditions are suitable.

Field 2 represents various types of bacteroid, such as found in *B. radicola* and other organisms of agricultural interest, found in cultures of the single strain of *B. typhosus* here studied. The motility of these bacteroid forms was frequently of the rotatory type, the two limbs of the Y at 28° C. revolving with great rapidity. On detachment of a limb this, whilst still short, moves in the ordinary way as does the ordinary bacillus, but as it lengthens it may take on a well-defined serpentine movement. Some of the bacteroids segment terminally, as shown, in oidal fashion, the small detached spheres being themselves highly motile.

Field 3 represents organisms apparently undergoing the plasmoptysis changes described by Fischer. A normal motile bacillus will sometimes be seen to extrude, anywhere in the bacillary axis, a minute spherical swelling. This gradually expands, the motility of the mother-cell being still unimpaired, progression being in the ordinary way, or by rhythmical serpentine movements. As the sphere expands the mother-cell, with its attached sphere, rapidly rotates, the sphere, if laterally placed, appearing to be presently swung off at a high velocity. Once detached the sphere exhibits a high degree of motility of its own. Its subsequent history is dealt with below. The mother-cell is meanwhile still motile, and may extrude a second, or even a third, new sphere which behaves as before. Sometimes, especially if a sphere has come into contact with another organism, the sphere will remain more or less motionless, whilst the mother-cell will revolve in all planes round the point of attachment to the sphere till finally separation is achieved. If these changes are watched in glucose broth on glucose agar, instead of in normal broth on plus 10 peptone agar, the spheres as formed, presumably on account of the rapidly rising acid tide, will expand and either burst (stained impression films show this well), or will gradually fade from view, either before detachment from the mother-cell, or after detachment, in either case losing their motility.

Field 4 represents types of organisms which are often figured in text-books as "involution" forms, especially those bearing a superficial resemblance to chlamydo-spores. There are also figured in this field two ovoids. All the forms here shown are highly motile.

Field 5 represents terminal and median segmentations of spherical form, occurring in bacilli, and mimicking oidal formation. The separated coccoidal bodies are motile.

*Field 6* shows bacillary, spherical and bacteroid forms undergoing chromatinolysis, the minute fragments of chromatin being frequently observed on the warm-stage in the process of extrusion from the mother-cell. Their subsequent development is dealt with below. Some of the extruded bodies are very minute, often measuring 0.1 to 0.2 microns in their greatest diameter, and able, as such, to pass the coarser filters such as Berkefeld's and Masson's. These minute bodies exhibit high degrees of motility.

*Field 7* shows various sizes of coccoidal bodies, mostly with thin walls, when the stain is taken relatively slightly, contrasting strongly with the thick-walled, deeply-staining coccoids, a few of which are seen in *Field 1*. Photographs of these two types of coccoids are seen in Part II of this paper. It is often said that these coccoid bodies are bacillary bodies seen in cross section. This view is the result of want of their observation on the warm-stage, when they may be readily observed to rotate, if pressure on them be sufficiently light, as definitely spheroidal bodies.

*Field 10* represents, grouped in one field, the various types of organism, which were often seen to be present at the same moment in a true microscopic field at the edge of warm-stage preparations of 4 per cent. glucose broth cultures on plus 10 agar. They were also often seen in subcultures in plus 10 broth from glucose cultures. In this field also are seen four forms resembling spirochaetes. These are large detached flagella.

*Field 11.* These spirochaetoid forms, often tapering to a fine point at each end, with wide spirals, varying in number from 4 to 5 to 70 and 80, the maximum counted (perhaps attached end to end), sometimes stretching across several fields, do not appear to be motile or to segment. They appear in fact to be flagella, attached forms being also shown. They may be seen on the warm-stage to adhere together one by one till thick tresses are formed, such as are sometimes seen in tetanus cultures. They are easily seen unstained, and have been noted in attachment to all the various types of organism shown, including the minute gonidial forms, except to the coccoid forms, and the thick-walled resting cells. The absence of flagella in these forms, appears, as will be seen in Part II, to be associated with non-agglutinability, their development into bacillary forms perhaps going hand in hand with the acquirement of flagella and of agglutinability. It is certain that when development from coccoid to bacillus is complete flagella are present, and that agglutinability is developed, but it has so far proved impossible to watch on the warm-stage the development of flagella *de initio*. In staining preparations of glucose cultures containing large numbers of flagella, attached and detached, it is not necessary to use silver salts. With a preliminary clearing with acetic acid and formalin beautiful preparations are readily made by counter-staining with carbol-fuchsin. In some preparations a fine reticulum may be seen of extremely fine short flagella in dense meshwork formation.

It is necessary to emphasize the fact that all the types of organism shown in Plate IV, except the thick-walled cells, and the large flagella, can frequently be noted in many, but not in all, quite young cultures of the *B. typhosus* in standard media, to which glucose has never been added, provided that patient search be made. It is also necessary to emphasize the fact that the various types of organism shown in Plate IV were again and again found in cultures from single cells, as well as in cultures from single colonies, the precaution always being taken continuously to watch development from single cell to single colony, for purposes of subsequent identification. The necessity for this precaution, and the uselessness in work of this nature of any method of single cell isolation which does not allow of continuous observation from single cell to single colony, will be readily understood by reference to *Field 6*, Plate IV, and *Field 8*, Plate IV. The presence of the minute gonidial bodies there depicted makes it impossible to be certain, in the attempt to isolate a single cell of normal bacillary form and size for example, that a minute gonidium is not at the same time being unconsciously picked up. If therefore the ordinary methods of isolation be employed, such as Barber's method, Malone's method, or the fragmented glass method, methods which



involve inoculation of a liquid medium without opportunity for adequate control observation of growth from single cell to single colony, it may happen, and does happen, that more than one organism is picked up, and that the experiment is ruined. If however a method be adopted which does allow of continuous observation to ensure that a given colony has developed from one cell, and from one cell only, the results obtained can be relied on. No satisfactory method of this nature was available, and one had therefore to be devised. This method is fully described in my paper on pp. 361—368 of this volume of *Journ. of Hygiene*.

## PLATES V AND VI.

These Plates reproduce drawings of continuous warm-stage observations of development from single organisms to single colonies.

In *Series A*, B, C, Plate V, and in *Series I*, Plate VI, is figured the development of bacteroids, unequal binary fission giving rise to ordinary bacilli which, as in Plate V A, may continue to divide by equal binary fission, or, as in Plate V D, again become triradiate, and again undergo unequal binary fission. As a rule only one limb of the Y is thrown off at a time, though segmentation of two or three limbs may go on simultaneously, or in short succession. It sometimes happens that a bacillus will undergo unequal fission without manifesting branching.

In *Series D*, Plate V, is seen the development of bacteroid forms from a diplococcal organism, the exact contour of which was determined by observation of rotatory movements *in situ* before elongation to bacillary form had begun.

In *Series G*, Plate VI, is seen development from a single coccoid form to ordinary bacilli.

In *Series H*, Plate VI, is seen development of a "chlamydosporoid" form, germination, segmentation of the new limb, separation, and germination of a fresh limb, with segmentation by equal binary fission of the limb first formed, being clearly seen. This was a highly motile organism, and no attempt was made to immobilize it in order to obtain a camera lucida drawing for fear of arresting its development, a free supply of oxygen having been found essential to rapid growth and segmentation of similar organisms. A freehand drawing was therefore alone possible. A similar course of events is shown in *Series J*, Plate VI.

In *Series E*, Plate VI, to which the same restriction applies, may be seen the plasmolysis phenomenon described by Fischer. In this case, however, continuous observation on the warm-stage enabled the observer to determine the fertility and motility of the organism in question, as depicted.

In *Series F*, Plate VI, is shown, at the end of the series, plasmolytic fragmentation of chromatin, followed by extrusion of chromatin, and by development of the highly motile extruded bodies into minute bacilli, each of these after a time exhibiting at each pole one of Artaud's nodes with a clear space between. These rapidly enlarged, though the enlargement is not drawn, under observation, still retaining their motility. These are the minute gonidial bodies, figured in Field 6, Plate IV, as present in, and extruded from, bacillary forms as well as from spheroidal, which can pass coarse bacterial filters, and which can sometimes be seen in standard media, as well as in glucose media, or in media to which HCl has been added, when the requisite alteration of concentration of solutes—in the direction of plasmolysis production—has been reached. And whenever very minute motile bacilli, especially when showing Artaud's nodes, appear in pure cultures of normal sized *B. typhosus* cultures it may safely be concluded that they have arisen in this way from minute extruded gonidial bodies. In order satisfactorily to study the development of these organisms a free supply of oxygen and of moisture is absolutely essential, no development of this nature taking place except at the moist edge of the preparation nearest to a free supply of air.

Having thus shown development from single cell to single colony it was then necessary to identify fully the colonies obtained. Eighteen different aberrant types were isolated, including giant bacillary forms, similar to those seen in Plate IV, Field 8, coccoid forms similar to those seen in Field 7, bacteroids similar to some of those seen in Field 2, chlamydo-sporoids similar to some of those seen in Field 4, sporangiods similar to those seen in Field 3, and resting cells similar to those seen in Field 1.

The broth cultures from each of these were fully identified by cultural, biochemical and serological tests, an agglutination of from 1/20,000 to 1/40,000 being in all cases eventually obtained.

In two cases identification was further completed by absorption tests, and in the case of the cultures handed to the War Office Committee of enquiry absorption and inoculation tests were also successfully passed.

During the course of the work undertaken in connection with study of methods of bacterial reproduction the following facts came chiefly into prominence. Some of these have already been noted by bacterial morphologists.

1. In some cultures of the lower bacteria, whether young or relatively old, whether standard in initial reaction, or containing glucose from which, in the case of the *B. typhosus*, acid is rapidly formed, or deliberately made acid by the addition of free HCl, aberrant forms of organisms have never been found, even after prolonged search. The entire population in such cases appears to consist of "normal" organisms, staining relatively lightly, and dividing by equal binary fission only. The general viability of such a population after a few days in 4 per cent. glucose cultures, or in HCl cultures with an initial reaction to phenolphthalein of plus 20, appears to be considerably lowered, as measured by subculture.

2. Aberrant types of organism are sometimes to be seen in small numbers in quite young cultures in "standard" media. These aberrant types, found in young cultures, sometimes in relatively large numbers, usually stain deeply and uniformly, unless undergoing chromatinolysis, when the fragmented chromatin appears as deeply staining dots, contrasting strongly with the less deeply-staining mother-cell. These aberrant types may be only aberrant in their deep receptivity of stain, there being no departure from the normal bacillus in outline. Other aberrant types, also characterized by uniformly deep receptivity of stain, may be branching or budding forms, or may be coccoid in form, or may simulate parasitic fungi by the formation of bodies resembling sporangia, chlamydospores and so forth. All these deeply-staining forms may be highly fertile.

3. These aberrant types, if already present in a standard culture, will increase in number with the age of the culture, or by the addition of glucose, or of free acid, the normal population under such circumstances beginning to disappear until it is again temporarily reinforced by direct descendants from these fertile aberrant forms. The viability of such a culture as a whole is greatly impaired by prolonged immersion in 4 per cent. glucose media, or in media containing an excess of free HCl. The viability of the deeply-staining

aberrant forms referred to is however higher than that of the general population, and it is clear that they have higher resisting powers to acid influence. This is also shown by the fact of their presence in greater number in old cultures than in young, and in the fact that on transference to fresh media the new generation appears to start from them and not from the survivors of the normal population. This can readily be demonstrated by watching the development of individual organisms on the warm-stage. It is quite unnecessary in order to establish the higher resisting powers to acid influence of these selected individuals to show also that they exhibit greater resistance to heat or to antiseptics than do "normal" individuals. This statement is necessitated by attempts that have unsuccessfully been made in the past to establish a higher degree of resistance to heat and to antiseptics in the case of so-called "involution" forms, as compared with normal forms, without any reference to the cardinal fact of their demonstrable fertility.

4. In addition to the fertile deeply-staining forms described there are also often to be seen in young cultures in standard media, as well as in glucose media, lightly-staining aberrant forms. These organisms, distinguishable from involution forms in their symmetry of outline, and in the fact that they do not fade from view on the warm-stage, may be themselves highly fertile. Examples of these in coccoid form are seen in Chart 4, Part II, of this paper.

5. On transference to standard media from glucose cultures, or from HCl cultures, aberrant forms tend rapidly to disappear, the need for their services being perhaps no longer required in early stages of growth on standard media. When, however, the age of these media increases, or when glucose is added, they again tend to reappear. And if careful search be made it will often be found, whilst the cultures are still young, or before glucose has been added, that deeply-staining bacillary forms are here and there present. So long as conditions remain more or less favourable these may divide by equal binary fission only. But when conditions become less favourable they may often be seen to take on unfamiliar methods of reproduction, though this, as shown in Plates V and VI, is also to be witnessed within an hour or two of transference to standard media.

6. Transference of genuine involution forms from old or otherwise unsuitable media to fresh standard media does not restore their uniform receptivity of stain, their symmetry of outline, nor their fertility. In some instances there may be seen to appear in genuine involution forms highly-refractile spherical bodies, suggesting droplets of lipoid material, which rapidly increase in size under observation. These bodies do not take the ordinary stains, but stand out as bright clear spaces in the irregularly stained matrix of the cell.

7. If standard broth media with an initial reaction of plus 10 to phenolphthalein be inoculated with acid-producing organisms, such as the *B. typhosus*, and be repeatedly titrated, evidence of a rapidly rising acid tide is forthcoming within two or three hours of incubation. A progressively unfavourable environment is thus rapidly produced which appears to be similar to, and to approxi-

mate to, the unfavourable environment of old cultures, or of cultures to which glucose or free acid has been added. The rapidly increasing concentration in a constant volume, with no provision for their removal, of degradation products from the substrate presented by the constituents of media, and by dead organisms, with a rapidly increasing concentration of organic catalysts no doubt accounts for this rising acid tide in standard media. In attempting therefore to estimate the reciprocal influence of bacteria and their surroundings in terms of morphological results it is obvious that the term "standard medium"—once inoculation has taken place, and once the population has begun greatly to increase—has no actuality.

#### CONCLUSIONS.

In the light of the facts here recorded it would appear that from the point of view of perpetuation of the lower bacteria through long periods of time, in laboratory cultures at least, the organisms usually regarded as "normal," normal, that is, in form, in their relatively slight capacity to retain the stain, and in their exclusive ability—exclusive so long only as conditions remain favourable—to divide by equal binary fission, represent the least important members of a total population. These "normal" organisms occur in the largest numbers in a given culture when the reaction to phenolphthalein approximates to the neutral point: and it appears that in some cultures they represent the entire population. When, however, as the result of rapid increase in numbers, the circumstances of life become more and more adverse the onus of carrying on the race appears to be chiefly laid on those deeply-staining highly fertile organisms which, from too ready an assumption of their sterility, have hitherto been indiscriminately ranged with genuine involution forms. From the point of view of perpetuation of the race as a whole these deeply-staining organisms, sometimes aberrant in form and sometimes not, able to produce fresh bacilli now by this method of reproduction, now by that, according to the osmotic needs of the moment, are incomparably the most important. In the history of bacteria of the lower orders it would appear that the existence of these deeply-staining organisms, and of the various types of reproductive activity which they exhibit, is a direct expression of the reciprocal influence through the ages of bacteria and their surroundings in terms of selective adaptation to the vicissitudes of bacterial life (unless indeed it be alternatively suggested that they represent a non-bacterial order, with equally dividing "bacteria" as a vegetative side-issue, as from their mimicry of the parasitic fungi one was tempted to surmise). And if it were not for these deeply-staining forms, with relatively high viability in acid media, it would be difficult to see how cultures of non-sporing bacteria could survive in the test-tube for long periods of time if reliance could only be placed on "normal" individuals with the lower degree of viability which is unquestionably imposed on them by prolonged residence in progressively adverse surroundings. It must not however be supposed that the process of selective adaptability is one which

can be followed at will in the laboratory and that "forms identical with the aberrant forms here depicted have arisen in definite response to the conditions experimentally provided, and may be expected constantly to reappear whenever the same environmental conditions are reproduced," as suggested by the Committee selected to study the facts presented. Because this is precisely what does not happen. As already stated these deeply-staining aberrant fertile organisms only appear to increase in cultures already containing them, as for example in cultures in which circumstance has deliberately been made adverse. They emphatically do not appear, within the limits of observation, in populations exclusively composed of normal organisms when the same adverse circumstances are introduced. One can only conclude therefore that in these selected individuals there resides, as the result of far distant training, the inherited faculty of reproduction by unfamiliar methods when necessity arises. And these methods are only unfamiliar because this has not been recognized, and because they have not been looked for.

NOTE.

This Committee consisted of Colonel Sir William B. Leishman, Colonel Professor J. G. Adami, Professor J. B. Farmer and Lieut.-Colonel D. Harvey.

The subject of enquiry was presented by me in a typed memorandum in the following terms:

"The sole objective of the present enquiry is determination of the complexity or otherwise of the life-cycle of the causal organism of Typhoid Fever, in so far as this is amenable to study in unfiltered laboratory cultures. We are therefore here concerned with one question only—Is the *B. typhosus*, or is it not, capable of being produced in any other way whatever beyond that of equal transverse binary fission of a pre-existing *B. typhosus*?"

During their work this Committee applied to my cultures the ordinary routine tests for purity, including absorption and inoculation tests. And they reported that they fully endorsed my statement that the cultures were pure cultures. They did not however apply the crucial test of purity which I had myself applied, namely that of isolation of single aberrant cells, and of continuous observation on the warm-stage of growth from single cell to single colony, with subsequent identification of the new races thus produced. They also fully confirmed by personal observation of warm-stage development my claim that the *B. typhosus* can be reproduced from non-bacillary forms by unequal binary fission, as well as from bacillary forms by the ordinary method of equal binary fission. Confirmation by independent observers of my main thesis, as specifically presented, was thus complete. It may therefore be unequivocally stated that it has now been definitely proved, and accepted, that the lower bacteria, as illustrated by the *B. typhosus*, can and do reproduce themselves in more ways than one, and that conse-

quently the reproductive life of this organism is not necessarily so simple as it has hitherto been assumed to be.

The Committee, I think wisely, deprecate the use of the term life-cycle in connection with the reproductive life of the individual bacterium as an organism which inevitably, sooner or later, as a necessary stage in the completion of its life-story must—in the case, for example, of a bacillus—pass through a non-bacillary phase. In the case of bacteria placed under the artificially favourable environment supplied for short periods of time by culture media with an initial standard reaction the life-story, such as it is, is unquestionably simple and not complex. But it is not sufficient to study the morphology of bacteria in the short periods when circumstance is easy. It must never be forgotten that this initial state of favourable circumstance is always fugitive in nature, in infected tissues, and in the test-tube, once the population has begun rapidly to increase in a confined space, with no provision for removal of degradation products. It is this progressively unfavourable environment which appears to have evoked a selective adaptation in virtue of which unfamiliar methods of reproduction come into play to ensure permanence of the race through long periods of time. To say therefore that in the lower bacteria the reproductive life is sometimes not simple but complex is indisputably true, because to preserve the race in terms of time a bacillus, for example, may have eventually to pass through a non-bacillary phase.

The confirmation, above referred to, by this Committee of the complexity of bacterial reproductive life under certain circumstances was however at once qualified by an evident reluctance to abandon the idea that the results demonstrated were the expression of an involutionary process, and had in consequence little or no bearing on the practice of bacteriological science.

In support of their view that involutionary processes could still be invoked to explain the results the Committee referred to the work of Fischer, and of Abbott and Gildersleeve, under the evident impression that the plasmoptysis of Fischer was looked upon by these authors as necessarily a pathological process. Fischer however, as well as Abbott and Gildersleeve, specifically state that they had had no opportunity of determining the fertility or sterility of the new bodies produced by plasmoptysis, a distinction which these authors clearly recognized to be one of fundamental importance. And their lack of opportunity of settling this point appears to have been due to the fact that they knew of no reliable method of isolating organisms for the purpose, a deficiency which I had been at great pains to make good. In the absence therefore of demonstration of the fertility of these organisms these authors had no alternative but provisionally to assume that they were sterile, and to conjecture that all degrees of plasmoptysis are therefore involutionary in nature. The bacteriologists of the Committee however in their official report make no reference to this qualification of Fischer's work by Fischer himself, and by Abbott and Gildersleeve, although they had my own positive evidence of fertility before them, which indeed they confirmed by their own observations.

In their reference therefore to this earlier work as confirmatory of their own view they must, doubtless under the stress of war work, either have failed to note that the crucial point of fertility had not been tested in this earlier work—this being the vital distinction between my work and that of previous observers, a distinction which they also omitted to record—or they had not recognized that demonstration of the fertility of new races abnormally produced is fatal to the involution theory, as generally understood. In either case, as they are careful to avoid the use of the word involution, it is necessary to point out that organisms undergoing pathological degrees of plasmolysis and plasmoptysis are only involution forms under another name, and that there appear to be physiological degrees of these phenomena resulting from disturbances of normal restraint of osmotic changes as well as pathological degrees of the same. It is indeed difficult to see how the occurrence of bacterial branching and budding, and of endogenous gonidia production, can be anything else but an expression of restrained plasmoptysis and plasmolysis, assuming, that is, that disturbance of the power of adjustment of osmotic variations within the cell and outside it is the true explanation of the various types of morphological aberrancy here illustrated—an assumption on which few, perhaps, would care at present to dogmatize.

## PART II.

### THE EFFECT OF THE COMPLEXITY OF THE REPRODUCTIVE LIFE OF BACTERIA ON THE AGGLUTINABILITY OF BACTERIAL EMULSIONS.

Belief in the ability of the non-sporing bacteria to divide under all circumstances by equal binary fission only is reflected in bacteriological practice, especially as regards determination of the precise etiology of infections, the methods employed for identification of bacteria, and the theory of the multiplicity of bacterial strains. It also enters largely into prophylaxis and treatment with specific serums and vaccines, and is an essential feature in the detection and control of bacterial carriers, as well as in epidemiological work, including statistical analyses. Now however that it is known that the life-story of bacteria, even in standard laboratory media, is one of great complexity, and that their reproductive life faithfully reflects the progressive changes of milieu inseparable from laboratory cultivation it becomes necessary to examine the various bacteriological problems referred to from a different point of view.

In the present communication I propose only to deal with the problem presented by serological reactions as an aid to identification of bacteria, and in doing so to show that it is hopeless to expect to obtain constant agglutination results unless due regard be paid to the morphological status of a bacterial population, both when used as an antigen for the production of agglutinin, and when tested for its power of provoking a specific reaction.

I propose, in other words, to show that necessary as attempts at standardization are in terms of initial reaction, or the opacity of an emulsion, or in terms of constancy of culture medium composition, one cardinal feature at least in standardizing serological reactions is determination of a standard morphological equivalent. Unless this be fully taken into account agglutination units have but little practical significance.

It has of course long been known that bacillary organisms of the lower orders of bacteria may occasionally take on a coccoidal phase, and that this may be associated with a deficiency in agglutinability by the specific serum. And there are well known to occur various vagaries of agglutinability, hitherto unexplained, such for example, as the loss of agglutinability on subculture to an agar slope, with subsequent recovery on again restoring the strain to broth. But, as a rule, the current conception of the simple life-history of bacteria has dominated the identification work of bacteriologists, and has prevented any systematic examination of the morphology of bacterial emulsions which might be expected to agglutinate with a given serum, but which nevertheless do not do so. And in consequence there has arisen, especially in bacillary dysentery, a heterogeneous collection of new "strains," marked by strange symbols, justification for the existence of at least some of which rests on the slenderest evidence. It is not possible as yet, owing to the bewildering complexity of the study of bacterial morphology, absolutely to prove that some of these many strains only represent different morphological phases of one strain, difference in form being associated with difference in antigenic values. Nor is it desired to insist that standardization in terms of morphology is the only cardinal factor to be considered in the standardization of bacteriological methods of serological aids to identification. But, as will be seen, there is abundant evidence to show that the morphological factor is one which demands the most searching study, which no bacteriologist can afford to set aside as unnecessary if the standardization of bacteriological methods generally is to be put on a scientific footing.

The value of the agglutination results here recorded entirely rests on the reliability of the technique employed. It is therefore necessary to give a series of control observations to show that constant results were uniformly obtained in using this technique when an emulsion of the same morphological equivalent was put up against its specific serum.

#### CONTROL CHART.

The technique employed in all cases was as follows. The method of reading was macroscopic, often confirmed by microscopic, or by hand-lens, examinations. Distribution of equal volumes of saline, serum and emulsion was made with a new graduated Pasteur pipette for each set of tubes used, the pipette being thoroughly washed and drained between each distribution of material. Dilutions in all cases began at 1/80, except when otherwise stated, the dilution in each case except the first being double that in the preceding tube. Twelve



hour cultures were uniformly employed, the test emulsions being put up without killing the organisms. In the case of *B. typhosus* cultures the plugged tubes were placed in a hot chamber for one hour at 56° C., after which time they were allowed to stand for one hour at room temperature when a first reading was taken, a second reading being taken 12 or 24 hours later. In the case of dysentery cultures the tubes were kept for 24 hours at 56° C. No attempt was made to standardize in terms of opacity, a deliberate omission in order to determine the effect of the omission on the control observations. As will be seen in Chart A the effect was nil. A control tube containing saline and emulsion only was put up for each set of observations in all the experiments.

The first set of observations deals with typhoid cultures. This set is divided into the following groups.

#### CHART 1, GROUP A.

In the case of strain Pierce, morphological variations in which formed the subject of Part I of this paper, it was found that the thick-walled coccoids there referred to, *vide* Plate VII, figure 1, were non-agglutinable. It was also found that under the influence of the acid produced from the glucose added to the cultures these thick-walled coccoids represented a resistant resting phase, and occurred in a bacillary population which was itself fully agglutinable. It was also found by isolation of one of these thick-walled cells that it germinated, and gave rise to large bacillary forms, which in their turn gave rise to forms which were again fully agglutinable. The process from agglutinable bacillus to non-agglutinable thick-walled cell and back again to agglutinable cell was therefore reversible. The results obtained are expressed in graphic form in Chart 1, which also gives the key to cultures submitted to agglutination tests, together with the dominant morphological equivalent for each culture. Photographs of these thick-walled coccoids are appended. It is noticeable that emulsions 4 and 8 were from glucose agar slopes.

#### CHART 1, GROUP B.

From the same strain Pierce subcultures from glucose broth were made as shown in the chart. In cultures 1 and 2 the dominant population was composed of thin-walled lightly-staining coccoids. Photographs of the actual emulsions of these coccoids used in these agglutination tests are given, together with a photograph of the mixed coccoid and bacillary emulsion 3, which agglutinated to 1/1280. The remaining emulsions 4, 5, 6 were mainly bacillary, as also was the original emulsion A, all of these agglutinating to high titre. It is again to be observed that the non-agglutinable emulsions 1 and 2 were from an agar slope, and from a glucose agar slope. Since recovery of agglutinability ensued on subculture in broth, *vide* cultures 4 and 5, associated with reversal to bacillary form, it might be thought that the appearance of a coccoidal non-agglutinable phase is the result of cultivation on a solid medium. That this is not necessarily the case is shown in Group C, where the non-agglutinable phase is seen to persist in broth culture.

#### CHART 1, GROUP C.

From the key to subcultures 1, 2, 3, 4, 5 from the same Pierce strain it will be seen, Chart 1, Group C, that the non-agglutinable coccoidal phase persists in broth cultures 2 and 5 with temporary reversion to agglutinable bacillary phase in broth culture 3, followed by return to coccoidal non-agglutinable phase on agar, culture 4: the same persistence of this in broth, culture 5, from the original agar, being again seen.

## CHART 1, GROUP D.

Having thus found in Groups A, B, C that, using the stock serum issued by the Lister Institute, a dominant coccoid population in a selected *B. typhosus* strain subcultured from glucose cultures, will show little or no signs of agglutination, whilst bacillary subcultures from single isolated coccoids (*vide* Plate V, Part I) may agglutinate in a dilution of 1/40,960, the same strain Pierce was then tested in parallel with an R.A.M.C. stock serum, and with the same Lister stock serum as before; two colonies being subcultured from the same agar plate, for purposes of comparison, into glucose broth, each tube of this being further subcultured as shown in the key. It was not found possible, at the date on which this experiment was carried out, to provide at the required moment a subculture from strain Pierce with as dominant a population of thin-walled coccoids as shown in Groups C and D. But a sufficient number were demonstrable to produce wide variations in the agglutination results obtained between the readings respectively of dominant coccoidal and dominant bacillary populations, with intermediate readings, discussion of which is for the moment deferred. In addition to this the following points of interest emerge from study of Group D.

A. Subculture 7, in which the coccoidal population was dominant, agglutinated only to 1/320 with both the Lister and the R.A.M.C. serums, in the case of colony A, and only to 1/160 with both serums, in the case of colony B. The amount in both the Lister and the R.A.M.C. serums of specific agglutinin to the coccoid phase of the *B. typhosus* would appear therefore to be very small. In the case of the Lister serum this had already strongly been suggested in groups A, B and C. The great similarity in the agglutination results in the case of subcultures 7, as regards both colonies A and B, also indicate that the course of development in the case of both these colonies, as shown in the particular subculture 7, was approximately the same. And this was confirmed by the dominant coccoid population in both. So far the distinction between coccoid non-agglutinable and bacillary agglutinable phases, already illustrated in groups A, B and C, appears to be a simple matter, when considered in the light of results obtained by using subcultures from a single colony tested against one serum.

B. When however further controls are established, as by testing subcultures from two colonies against two serums in parallel, most discordant results emerge, which suggest that it is not merely a question of the relative percentages of coccoidal and bacillary members of a population. For example, subculture 10, colony A, gives a full titre of 1/20,480 with both Lister and R.A.M.C. serums, whilst subculture 10, colony B, gives a full titre of 1/20,480 with Lister serum, but a titre only of 1/1280 with R.A.M.C. serum. Again, subcultures 1, 2, 4, 6, 8, 9, colony A, give full titre with Lister serum, but much smaller titres with R.A.M.C. serum: whilst, allowing for the smaller number of subcultures tested in the case of colony B, there is again great similarity in the results obtained with Lister serum, but great disparity in the results obtained with R.A.M.C. serum. Imperfect observation of the agglutination results affords one way of escape from the dilemma. This is however excluded by the control results given at the opening of Part II of this paper. Contamination of the cultures affords a second way of escape. This however is practically excluded by the fact that cultures 1, 2, 4, 6, 8, 9, 10 agglutinated to 1/20,480 with Lister serum in the case of colony A, and that cultures 1, 2, 8, 9, 10 agglutinated to the same titre with the same serum in the case of colony B. Still a third way of escape is offered by the theory, upheld by many, that any given bacterial culture may be composed of numerous strains, and that some subcultures from such cultures may exalt this strain, and other subcultures that strain. This way of escape also appears to be closed by the fact, demonstrated in Part I, that organisms appearing to represent different strains in one culture can develop within a few hours from a single cell. The true explanation in fact appears to lie in this demonstration, though it is not suggested that other factors, as yet

undetermined, may not be partly responsible for the agglutination vagaries here illustrated. Confirmation of the view that each agglutination departure from full titre corresponds with a definite morphological equivalent was obtained by careful examination of subcultures 1, 2, 4, 6, 8, 9, 10 when it was found that:

- (1) Repeated replating gave no indication of contamination.
- (2) Culturally, biochemically and serologically (in terms of Lister serum) they were genuine *B. typhosus* cultures.

(3) Morphologically some of the cultures contained in addition to "normal" bacilli large numbers of forms of bewildering variety, the relative percentage numbers of these—which were mostly bacillary—varying with each subculture. In addition, for example, to coccoids and to "normal" organisms would be seen bacilli considerably larger, and very much smaller, than the normal, some exclusively bacillary, some cocco-bacillary, others undergoing budding or branching, others again undergoing chromatinolysis. Some had pointed ends, others had rounded ends: some were small, exhibiting Artaud's nodes, others were considerably larger, also exhibiting Artaud's nodes. Some were thick-walled bacilli, taking the stain deeply, others, again, of the various types described took the stain relatively lightly.

The amount of work involved in attempting to obtain an accurate idea of the relative proportion of each of these various types in a large number of cultures exhibiting intermediate degrees of agglutinability would clearly be prohibitive, without a trained staff of workers. No attempt therefore was made in the observations now to be recorded to interpret in morphological terms varying degrees of agglutinability of different subcultures of the same culture when tested either with one serum, or with two or more serums in parallel: the main object being merely to show that standardization in terms of morphology must in the future be comprehensively studied if constantly reliable results are to be obtained.

#### CHART 1, GROUP E.

That some serums appear to have been unconsciously prepared with polyvalent antigens, polyvalent that is in the sense of polyvalency of morphological types and not in the sense of polyvalency of "strains," is shown by Group E. In this group subcultures from the same strain Pierce were tested against the Oxford stock serum, and against the R.A.M.C. serum. The results, as before, with the R.A.M.C. serum are poor, whilst the results with the Oxford serum, against the Pierce strain, are very good, suggesting lack of morphological polyvalency in the former serum, and its presence in the case of the latter serum.

So far tests have been confined to subcultures of the strain of *B. typhosus* from glucose cultures, which appear to be particularly favourable to the initiation of different types of morphological development. To this extent therefore the tests so far applied would appear to be unnecessarily severe, and perhaps of little practical value unless confirmed by similar results obtained from tests applied to subcultures from ordinary cultures to which glucose has not been added. Tests were therefore applied to subcultures from ordinary cultures, in order to determine if similar vagaries of agglutination occurred, with the following results.

#### CHART 2, GROUP F.

Here considerably better results are obtained, though even with such good serums as the Oxford and Lister serums marked variations in the titres given with each of these occur. Taking the results given with the three serums the minimum occurs with the R.A.M.C. serum of 1/640, and the maximum with the Lister serum of 1/40,960.

## DYSENTERY CULTURES.

## CHART 3, GROUP G.

As shown in this chart four subcultures from 12 hour agar cultures of Lister stock strains F and Y were tested respectively against Lister F and Y stock serums, cross tests being also carried out. In this experiment the condensation water in each agar tube was replaced by broth, cultures 2 and 4 representing the broth substitute for condensation water in cultures 1 and 3. The object of this arrangement was merely to show that different morphological results, with different morphological readings, can be shown in one test-tube according to whether the agar slope, or the broth at the bottom of the tube, be inoculated at the same moment from the same source: care of course being taken to keep the tubes vertical during incubation, and to ensure that in removing the broth culture no admixture of this with the culture on the surface of the agar slope not covered by broth takes place. This agar culture, after removal of the broth, was itself removed with a sterile glass rod from the upper half only of the slope, and was then emulsified in sterile broth: both cultures being then at once tested. Study of the chart shows that:

1. The *B. Flexner* broth subculture 4 does not agglutinate at all, in any of the given dilutions, either with Flexner serum, or with Y serum: whilst the B. Y broth culture 4 agglutinates to 1/160 with Flexner serum, and to 1/320 with Y serum. The approximate respective morphological picture in each of these cultures is given in the chart.

2. The *B. Flexner* agar subculture 3 agglutinates to 1/640, with Flexner serum, and to 1/160 with Y serum: whilst the B. Y agar subculture 3 agglutinates to 1/320 with Flexner serum, and to 1/320 with Y serum.

3. *B. Flexner* agar subculture 1 agglutinates to 1/160 with both Flexner serum and Y serum, whilst B. Y agar subculture 1 agglutinates to 1/640 with Flexner serum, and not at all with Y serum.

4. *B. Flexner* broth subculture 2 does not agglutinate at all, in any of the dilutions, with Flexner serum, but to 1/1280 with Y serum: whilst B. Y broth subculture 2 does not agglutinate at all, in any of the given dilutions, with Flexner serum, and to 1/640 with Y serum.

Thus, to take an extreme example, agglutination of B. Y agar subculture 1 would suggest that this was a culture of *B. Flexner*, other tests being in the same direction, the figures for agglutination being 1/640 with Flexner serum, and nil with Y serum: whilst agglutination of B. Y broth subculture 2 would suggest that this was a culture of B. Y, the figures being nil with Flexner serum, and 1/1640 with Y serum. Taking the agglutination results of all four subcultures as a whole an empirical agglutination mean, E.A.M., can be obtained for each set of four observations with one serum by dividing the total length of agglutination lines by the number of subcultures examined.

The results may then be expressed:

<i>B. Flexner</i> versus Flexner serum = 10 E.A.M.			
"	"	Y	" = 13 "
B. Y	"	Flexner	" = 16 "
"	"	Y	" = 13 "

The agglutination results, in other words, suggest that, taking the subcultures as a whole, *B. Flexner* agglutinates better with Y serum than with its own serum, and that B. Y agglutinates better with Flexner serum than with Y serum.

Unfortunately reliable sets of sugars were not available when these experiments were carried out: so no opportunity occurred of determining if the results obtained were associated with any biochemical variations. Observations since undertaken, indicating in some cases association of biochemical variation with serological, will be published separately.

## CHART 3, GROUP H.

Observations were then carried out with R.A.M.C. subcultures from stock Flexner and Y cultures, kindly supplied by Lt.-Colonel Harvey, tested against R.A.M.C. stock Flexner and Y serums.

The massed results obtained with six subcultures, instead of four, show a better specificity of reaction in the case of R.A.M.C. cultures and serums than in the case of Lister cultures and serums, the figures being:

<i>B. Flexner</i> versus Flexner serum = 15 E.A.M.				
"	"	Y	"	= 10 "
B. Y	"	Flexner	"	= 6 "
"	"	Y	"	= 17 "

A considerable variation is, however, to be noted in each group between the minimum and the maximum dilutions at which agglutination occurred.

## CHART 3, GROUP I.

The R.A.M.C. stock *B. Flexner* culture used for the observations recorded in Group G, was kept in a sealed tube for over three months at 5° C., and was again subcultured as shown in Group H, several replating observations being made to ensure the absence of contamination. The object of the experiment was to determine the effect of subcultivation from an old culture in the direction of inducing in these subcultures a greater, or a lesser, variation in their agglutination figures than in the case of subcultures from young cultures, as in Group G. At the same time it was desired to test these subcultures against a Lister Flexner serum, and against a Lister F Y serum.

For purposes of comparison the figures from A in Group G are reproduced in the present Group H. The E.A.M. for the R.A.M.C. Flexner subcultures tested with R.A.M.C. Flexner serum is now seen to rise from 15 to 42, the maximum titre supplied by the makers being greatly exceeded in the case of cultures 1, 3, 2, 6. The E.A.M. for the same subcultures tested with Lister Flexner serum C 94 is only 5, three of the subcultures giving no reaction at all. The E.A.M. for these subcultures tested with Lister Flexner Y serum, is the much better figure 32, which is of particular interest in view of the fact that this serum appears to have been prepared by inoculation of F and Y antigens, Lister F and Y cultures having been shown in Group A to be more or less interchangeable.

The maximum variations, as for example B—between 1/160 and 1/20,480, again show how nearly subculture 4, for example, came to the minimum diagnostic titre, even when tested against so good a Flexner serum as R.A.M.C. serum F 7.

CHART 3, GROUPS K<sub>1</sub>, K<sub>2</sub>, K<sub>3</sub>.

This chart illustrates the discordant results obtained when the R.A.M.C. stock Flexner culture was replated, half of a colony being subcultured on to agar, the other half being subcultured into broth, at the same moment. The same Flexner culture was replated eight times, each time on a series of five plates. Each series was lettered in succession, A, B, C, D, E, F, G, H, a single colony from plates 4 or 5 from each series C, D, E, F, G, H being divided as described. There was an interval of three days between each replating, the original culture remaining at room temperature in the interval.

CHART 4, GROUP L<sub>1</sub>.

This chart illustrates the results obtained by testing subcultures, for the most part at random—and not entirely, as hitherto, in groups, from a Y culture, obtained from the Kitchener Hospital, Brighton, against different Y and F Y serums, and against a polyvalent serum. As will be seen the agglutination figures obtained do not, taken as a whole,

in this case indicate a marked advantage in favour of the R.A.M.C. polyvalent serum employed, wide variations, from 1/160 to 1/10,240, of readings occurring, in the case of five subcultures from the original agar when tested with this serum.

#### CHART 4, GROUP L<sub>2</sub>.

In this experiment a normal polyvalent serum, R.A.M.C. polyvalent Flexner Y serum D<sub>1</sub> was tested against:

- a. The same Y strain as used in Chart 4, L<sub>1</sub>.
- b. A second Y strain, also from the Kitchener Hospital.
- c. The stock R.A.M.C. Flexner strain, Ledingham, employed in the earlier experiments.

It was desired to determine what variation, if any, occurred when a different medium was employed, such as the tryptic agar medium recommended for meningococcus work.

As will be seen the results obtained with this polyvalent F Y serum were very good, and more constant. The results obtained with agar and with tryptic agar subcultures are also very similar, indicating that the change of medium has little or no effect in producing different degrees of agglutinability.

#### CHART 5, GROUP M.

Attention was now turned to a stock Shiga-Kruse culture, kindly supplied for the purpose by Professor Dreyer's staff at Oxford, subcultures from this being tested against a stock R.A.M.C. Shiga serum, and a stock Lister Shiga serum.

The results obtained may be summarized as follows:

##### 1. *With Oxford Shiga serum.*

- (a) Subcultures 13, 14, 15 give a uniform figure of 1/40,960, suggesting that this is an ideal serum.
- (b) Subcultures 19, 20, 21 from the original agar slope—which had been standing for four days at room temperature—now uniformly declined to agglutinate in a higher dilution than 1/80.
- (c) Subculture 25 from subculture 19 still persisted in refusing to agglutinate, whilst subculture 28 from subculture 20 agglutinated in a dilution of 1/2,560, subculture 27 from subculture 21 now agglutinating in a dilution of 1/40,960.
- (d) Subcultures 31 and 32 from the original agar slope—which had been standing for six days at room temperature—now both agglutinated in dilutions of 1/20,480.

##### 2. *With Lister serum.*

- (a) Subcultures 13, 14, 15 which all gave a figure of 1/40,960 with Oxford serum gave with Lister serum figures of 1/80, 1/160 and 1/320, suggesting that the phases agglutinable by Oxford serum are not represented by specific agglutinins in Lister serum.
- (b) Subcultures 25, 27, 28 now agglutinate to 1/10,240 with Lister serum, suggesting that agglutinins specific to the phases agglutinated by Lister serum are absent in Oxford serum, in so far as subculture 25 is concerned, and are partly absent in so far as subculture 28 is concerned.

##### 3. *With R.A.M.C. serum.*

Similar results as with Oxford serum, and with Lister serum.

From the table showing the maximum variations obtained with each serum it appears that of eleven subcultures tested respectively with R.A.M.C. serum, and with Oxford

serum, three subcultures in the first case, and four in the second case, failed to give any diagnostic readings whilst with the Lister serum two subcultures out of six failed to give reliable diagnostic readings.

#### CHART 6, GROUP N.

Attention was finally turned to *Meningococcus*, Type 1, subcultures from this "strain" being tested in parallel, *vide* Chart 6, against the four Type serums. If the morphology table is referred to, together with the serological table, it will be seen that the results obtained are suggestive. The acquisition by one "strain" of agglutinability by meningococcal serums specific to other "strains" has more than once in recent months been noted, though no satisfactory explanation has been forthcoming. The results shown in Chart 6 suggest however that the explanation lies in the existence of a morphological equivalent for each so-called strain of meningococci, and that the four meningococcal "strains" (meningococcal and parameningococcal) represent developmental phases of one strain only. That the developmental changes to be seen in meningococcal cultures are as complex as those demonstrated by warm-stage observations in Part I of this paper in the case of organisms of the enteric group I fully demonstrated in 1917, also by warm-stage observations (*vide Brit. Med. Journ.* Sept. 22, 1917). That one was perhaps then misled into regarding the unquestionable occurrence of budding, and of endofragmentation of chromatin, in the so-called giant-cells as indications that these organisms belong to the parasitic fungi does not alter the basic fact of demonstration of the fertility of the buds and of the fragments of chromatin thus produced. Nor does this affect the further fact that meningococci were shown on the warm-stage to be produced both vegetatively from pre-existing meningococci, and by chromatinolysis from giant-cells which had till then been believed to represent sterile involution forms. And the fact that in the case of the enteric group of organisms some morphological phases of development have in Part I of this paper been indisputably proved to be associated with agglutination vagaries lends collateral support to the direct evidence produced in Chart 6 that what is true of the enteric organisms is also true of the meningococcus. It appears to be a reasonable view that in the case of the enteric organisms the occurrence of budding and of branching, and of the production of gonidia, is an expression of physiological degrees respectively of plasmoptysis and plasmolysis, operating even in young standard media. And this appears also to be a reasonable explanation of the occurrence of budding in "giant" cells in meningococcal cultures under environmental conditions favourable to the induction of moderate degrees of plasmoptysis, and of the occurrence of gonidia production from chromatinolysis in the "giant" cells under conditions favourable to the induction of moderate degrees of plasmolysis. And it is noteworthy that this chromatinolysis in giant-cells in meningococcal cultures is best seen in media containing serum. It is also to be noted, emphasis being laid upon this point in the case of the enteric organisms, that these unfamiliar methods of reproduction have not been noted in cultures which appear to be exclusively composed of normal populations, the ordinary vegetative forms appearing not to depart from the ordinary methods of reproduction by equal binary fission only, even when conditions are markedly favourable for unusual types of reproduction. In the case of meningococcal cultures it appears possible that the explanation of the fact that some cultures will not easily survive, whilst others readily survive, is due to the exclusive presence of vegetative forms in the former case, and in admixture with fertile giant-forms in the second case, these being able to survive adverse circumstance in consequence of increased resistance to such circumstance, as was found to be the case with organisms of the enteric group.

## CONCLUSIONS.

There appears, from these observations, to be no doubt that changes in agglutinability are often associated, in the case of the organisms examined, with the occurrence of developmental changes in such organisms, changes which have in many cases definite morphological values. It must however not be supposed that a claim is here put forward that a different morphological equivalent exists for each of the agglutination vagaries here disclosed. The subject is far too complex a one for any such claim to be made. The evidence so far produced is that of a preliminary investigation only, and is published now in the hope that other workers will devote their attention to the morphological problems dealt with. In the meanwhile an attempt is being made to correlate the results obtained with the results of testing subcultures from single cells, and to show that reliable polyvalent serums can be obtained by construing valency in terms of morphology and not in terms of "strains" unrelated to developmental phases. It will be evident that absorption tests are of little value in this work unless the morphological equivalent of each emulsion used in the preparation of a serum is first worked out.

## NOTE.

As further evidence of the reliability of the macroscopic method employed throughout these observations the subjoined figures of control titration tests may be cited, the test which gave the largest error being selected. The maximum experimental error in this test is seen to be 5.3 per cent., as gauged by titration of N/20 HCl with N/20 NaOH, each tube in the total series of 10 tubes receiving measured quantities of HCl in water as follows. First filling: equal volumes of acid in all 10 tubes. Second filling: tubes 2 to 9 receive half the volume of the preceding tube, tube 1 receiving a full volume of acid, tube 10 receiving none. Third filling: each tube receives a fresh volume of acid equal to the volumes employed in the first filling. In this way the distribution of saline, serum and emulsion was reproduced as closely as possible, with the result that a severe test of accuracy was imposed with regard to the thirty measurements employed.

In 6 of the 10 tubes 2.90 c.c. of N/20 NaOH were required for neutralisation.

„ 2	„	2.70 c.c.	„	„	„
„ 1	„	2.82 c.c.	„	„	„
„ 1	„	2.85 c.c.	„	„	„

In this control test therefore we get a mean of 2.80, a mode of 2.90, and a maximum experimental error of 5.3 per cent.

It has of course long been known that inconstant serological readings may occur when observations are made with different emulsions made from one living culture at different intervals of time, using the same serum throughout. In many of the observations recorded in the text it is shown that wide



variations may occur when a series of fractions of the same emulsion from one culture are put up at the same time against different serums specific to the organism under examination, the existence of multiple strains being usually regarded as adequate explanation of such variations, the absence of considerable experimental error being assumed. The control observations given in the text and in this note show that in the case of the variations here recorded the experimental error can be excluded.

In view of the fact demonstrated in the text that wide morphological variations may occur in living fertile descendants of a single cell, some of these variations being definitely associated with wide variations in serological equivalents in terms of one serum, it would appear that the multiple strain theory is here inadequate. And this view is confirmed by the following transitions occurring within a few hours.

At 10.0 a.m., 13. ii. 18, an emulsion was made in plus 10 broth from a 13 hour 4 per cent. glucose agar culture inoculated with a pure culture of *B. typhosus*, and a fraction of the living emulsion was tested at once (*vide* A) against Lister *B. typhosus* serum: C 105: 28. xi. 17: 1/20: 1/6000.

At 2.30 p.m. on the same day a second fraction of the same living emulsion in broth was, after incubation at 37° C. for 4½ hours, tested against the same serum from the same tube (*vide* B).

Twelve hours later a third fraction of the same living emulsion in broth was, after incubation at 37° C. for 16½ hours, tested against the same serum from the same tube (*vide* C).

In each case a reading was taken after one hour at 56° C., and again after a further eight hours at 56° C. There was no difference in the two readings in any tube.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	Control	
nil	nil	nil	nil	nil	nil	nil	nil	nil	... A
C A	C A	C A	C A	C A	nil	nil	nil	nil	... B
C A	C A	C A	C A	C A	C A	C A	C A	nil	... C

C A = complete agglutination

Morphology of A	...	exclusively coccoid
"	B	... mixed coccoid and bacillary
"	C	... bacillary population predominant

The transition from non-agglutinable to agglutinable, with corresponding morphological transition, is however not necessarily rapid, a more or less stable condition of non-agglutinability being sometimes met with which, in one case, lasted five days in spite of repeated subculture in broth. In such case the return of agglutinability, and of bacillary form, sometimes only takes place on subculturing to agar from broth, the inverse of the usual experience.

The work on which Part I of this paper is based was carried out by me in the laboratory of the Addington Park War Hospital. The work on which

Part II is based was undertaken in the Central Laboratory of the Kitchener Hospital, Brighton. In both cases the work was the outcome of previous work on bacterial morphology carried out by me as Director of the Constance Trotter Research Fund in a laboratory generously placed at my disposal by the Governing Body of the Lister Institute.

To my laboratory attendant, E. Hawkins, I am greatly indebted for scrupulous care in the preparation of media and other material.

#### NOTE EXPLAINING CHARTS.

In the following charts are given:

1. The serum dilutions employed, each dilution except the first being double that of the preceding dilution.

In all cases the end-points, denoting partial agglutination, are given, represented by the symbol +. The end-points of complete agglutinations, and symbols representing the numerous gradations between complete agglutination and that just visible with the aid of a hand-lens, are deliberately omitted so as not to confuse the issue.

In determining the end-points here recorded extreme care was taken, in each series, to study the gradations between each tube in a row of nine tubes in relation to the gradations between the tubes in all the rows. The symbol 0 represents complete absence of agglutination.

2. The numbers of each subculture, generally with the dates of subculture.

3. Keys giving the "genealogy" of the subcultures.

4. The particulars of each serum employed, as to source, date of preparation, dilution and reputed maximum titre.

5. Whenever possible, the morphological equivalent.

In Chart A the brackets opposite the numbers 3, 4, 5=6, 7, 8=11, 12=13, 14, 15, 16=1, 2, 3, 4, indicate that each group of numbers indicates identical fractions of one emulsion (*vide* NOTE).

In number 2, of date 11. I. 18, the final dilution of 10,240 was inadvertently omitted.

#### CHART A.

##### CONTROL OBSERVATIONS SHOWING RELIABILITY OF AGGLUTINATION TECHNIQUE EMPLOYED (a) *B. TYPHOSUS*, (b) *B. DYSENTERIAE*.

Morphological equivalent of each control emulsion "normal" bacilli.

##### *B. typhosus*.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	Control	Date	Number
+	+	+	+	+	+	+	+	0	10. I. 18	1
+	+	+	+	+	+	±	in last tube	0	11. I. 18	2
+	+	+	+	+	+	+	+	0	18. I. 18	3
+	+	+	+	+	+	+	+	0	"	4
+	+	+	+	+	+	+	+	0	"	5
+	+	+	+	+	+	+	+	0	5. II. 18	6
+	+	+	+	+	+	+	+	0	"	7
+	+	+	+	+	+	+	+	0	"	8
+	+	+	+	+	+	+	+	0	8. II. 18	9
+	+	+	+	+	+	+	+	0	11. II. 18	10
+	+	+	+	+	+	+	+	0	28. II. 18	11
+	+	+	+	+	+	+	+	0	"	12
+	+	+	+	+	+	+	+	0	1. III. 18	13
+	+	+	+	+	+	+	+	0	"	14
+	+	+	+	+	+	+	+	0	"	15
+	+	+	+	+	+	+	+	0	"	16

Lister *B. typhosus* serum used throughout. Dilution 1/20: titre "maximum" 1/6000: dates 11. IX. 17: 28. XI. 17, etc.

*Reproduction of Aerobic Bacteria*CHART A—*contd.*

1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12,800	Control	Date	Number
+	+	+	+	+	+	...	...	0	1. XI. 18	1
+	+	+	+	+	+	...	...	0	"	2
+	+	+	+	+	+	...	...	0	"	3
+	+	+	+	+	+	...	...	0	"	4

R.A.M.C. Polyvalent serum = *B. dysenteriae* ("B. Y").

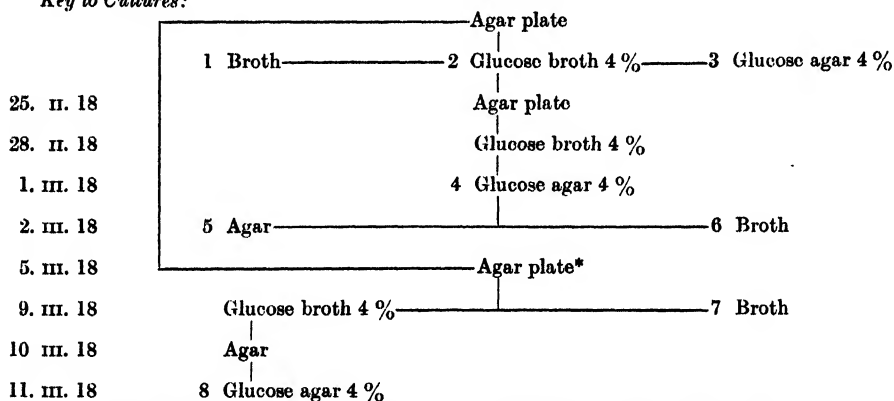
CHART 1, GROUP A.

*B. typhosus*, Strain Pierce versus Lister *B. typhosus* Serum C 105: 1/20: 1/6000:  
28. XI. 17.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	Cultures	Morphology
+	+	+	+	+	+	+	+	1	Dom. pop. bacillary
+	+	+	+	+	+	+	+	2	Dom. pop. bacillary
+	+	+	+	+	+	+	0	3	Dom. pop. bacillary
0	0	0	0	0	0	0	0	4	D.P. thick-walled cocc.
+	+	+	+	+	+	+	+	5	D.P. bacillary
+	+	+	+	+	+	0	0	6	D.P. bacillary
+	+	+	+	+	+	+	+	7	D.P. bacillary
0	0	0	0	0	0	0	0	8	D.P. thick-walled cocc.

All control tubes negative.

## Key to Cultures:



\* This indicates a fresh plating on 5. III. 18 direct from original agar plate.

CHART 1, GROUP B.

*B. typhosus*, Strain Pierce versus Lister *B. typhosus* Serum C 105: 1/20:  
1/6000: 18. I. 18.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cult. Morphol.
0	0	0	0	0	0	0	0	0	0	2 Coccoidal
+	+	+	0	0	0	0	0	0	0	1 Coccoidal
+	+	+	+	+	0	0	0	0	0	3 Cocco-bacill.
+	+	+	+	+	+	+	+	0	0	A Bacillary
+	+	+	+	+	+	+	+	+	0	5 Bacillary
+	+	+	+	+	+	+	+	+	+	4 Bacillary
+	+	+	+	+	+	+	+	+	+	6 Bacillary

All control tubes negative.

**Key to Cultures (Chart 1, Group B):**

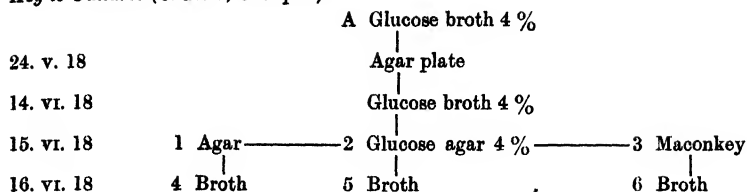


CHART 1, GROUP C.

*B. typhosus*, Strain Pierce versus Lister *B. typhosus* Serum C 105: 1/20:  
1/6000: 6. VI. 18.

[illegible]

All control tubes negative.

**Key to Cultures:**

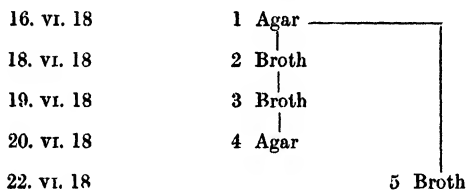


CHART 1, GROUP D.

*B. typhosus*, Strain Pierce *versus* Lister *B. typhosus* Serum and R.A.M.C.  
*B. typhosus* Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Cultures	Serum Colony	Cont.
+	+	+	0	0	0	0	0	0	7	Lister A 1	0
+	+	+	+	+	+	+	0	0	5		0
+	+	+	+	+	+	+	+	0	3		0
+	+	+	+	+	+	+	+	0	1, 2, 4, 6, 8, 9, 10		0
+	+	+	0	0	0	0	0	0	7, 9	R.A.M.C. A 2	0
+	+	+	+	0	0	0	0	0	1		0
+	+	+	+	+	0	0	0	0	2, 5, 6,		0
+	+	+	+	+	+	0	0	0	4		0
+	+	+	+	+	+	+	0	0	3, 8		0
+	+	+	+	+	+	+	+	0	10		0
+	+	0	0	0	0	0	0	0	7	Lister B 1	0
+	+	+	+	+	+	+	+	0	1, 2, 8, 9, 10		0
+	+	0	0	0	0	0	0	0	7, 9	R.A.M.C.	0
+	+	+	+	0	0	0	0	0	1		0
+	+	+	+	+	0	0	0	0	2, 10		0
+	+	+	+	+	+	0	0	0	8		0

CHART 1, GROUP D—contd.

MAXIMUM VARIATIONS IRRESPECTIVE OF WHICH SERUM IS EMPLOYED.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Cultures	Colony
+	+	+	...	...	...	...	...	...	7, 9	A 1, A 2
+	+	+	+	...	...	...	...	...	1	
+	+	+	+	+	...	...	...	...	2, 5, 6	
+	+	+	+	+	+	...	...	...	4	
+	+	+	+	+	+	+	...	...	5, 3, 8	
+	+	+	+	+	+	+	+	...	3	
+	+	+	+	+	+	+	+	+	1, 2, 4, 6, 8, 9, 10	
+	+	...	...	...	...	...	...	...	7, 9	B 1, B 2
+	+	+	+	...	...	...	...	...	1	
+	+	+	+	+	...	...	...	...	2, 10	
+	+	+	+	+	+	...	...	...	8	
+	+	+	+	+	+	+	+	+	1, 2, 8, 9, 10	

Key to Agglutinations:

- A 1. *B. typhosus* Colony 1 v. Lister serum C 105 : 1/20 : 1/6000 : 23. viii. 18.
- A 2. *B. typhosus* Colony 1 v. R.A.M.C. serum : 1/20 : 1/7000 : 18. ix. 18.
- B 1. *B. typhosus* Colony 1 v. Lister serum C 105 : 1/20 : 1/6000 : 23. viii. 18.
- B 2. *B. typhosus* Colony 1 v. R.A.M.C. serum : 1/20 : 1/7000 : 18. ix. 18.

Key to Cultures:

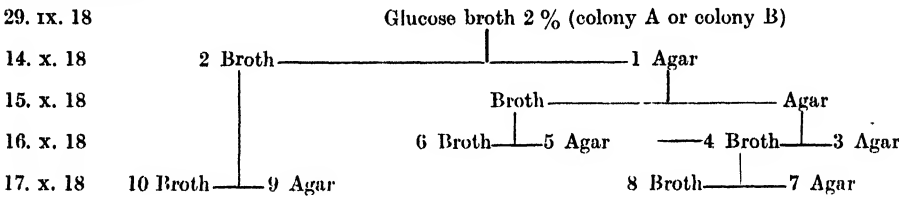
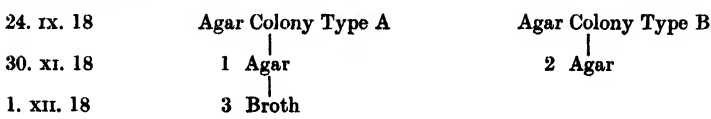


CHART 1, GROUP E.

*B. typhosus*, Strain Pierce versus R.A.M.C. *B. typhosus* Serum and Oxford *B. typhosus* Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cult.	Serum	Cont.
+	+	+	+	+	+	+	+	+	+	1	Oxford	0
+	+	+	+	+	+	+	+	+	+	2		0
+	+	+	+	+	+	+	+	+	+	3		0
+	+	+	0	0	0	0	0	0	0	1	R.A.M.C.	0
+	+	+	+	0	0	0	0	0	0	2		0
+	+	+	+	+	+	0	0	0	0	3		0

Key to Cultures:



## CHART 2, GROUP F.

*B. typhosus*, Oxford Strain Edwin *versus* Oxford Serum, Lister Serum and R.A.M.C. Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cultures	Serum
+	+	+	+	+	0	0	0	0	0	11	Oxford
+	+	+	+	+	+	0	0	0	0	5	
+	+	+	+	+	+	+	0	0	0	4, 6, 10	
+	+	+	+	+	+	+	+	0	0	12	
+	+	+	+	+	+	+	+	0	0	5, 6, 11	Lister
+	+	+	+	+	+	+	+	+	0	4, 12	
+	+	+	+	+	+	+	+	+	0	10	
+	+	+	+	0	0	0	0	0	0	12, 6	R.A.M.C.
+	+	+	+	+	0	0	0	0	0	11, 5	
+	+	+	+	+	+	0	0	0	0	10	
+	+	+	+	+	+	+	0	0	0	4	

## MAXIMUM VARIATIONS IRRESPECTIVE OF WHICH SERUM IS EMPLOYED.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cultures
+	+	+	+	...	...	...	...	...	...	12, 6
+	+	+	+	+	...	...	...	...	...	11, 5
+	+	+	+	+	+	...	...	...	...	10, 5
+	+	+	+	+	+	+	...	...	...	4, 6, 10
+	+	+	+	+	+	+	+	...	...	12, 5, 6, 11
+	+	+	+	+	+	+	+	+	...	12, 4
+	+	+	+	+	+	+	+	+	+	10

Key to Cultures:

					Agar (mainly slender filaments with minute buds)	21. XI. 18
4	Agar	5	Tryp. agar	6	Broth	22. XI. 18
10	Broth	11	Broth	12	Agar	23. XI. 18
16	Agar	17	Agar	18	Broth	Not tested
All control tubes negative.				Stock Oxford culture despatched 20. XI. 18 : received 21. XI. 18.		

## CHART 3, GROUP G.

*B. dysenteriae*: Lister Stock Flexner Culture and Lister Stock Y Culture *versus* Lister Stock Serums B. Flexner and B. Y.

E.A.M.	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control	Cultures	Experiment
10	0	0	0	0	0	0	0	0	2, 4	A
	+	+	0	0	0	0	0	0	1	
	+	+	+	0	0	0	0	0	3	
13	0	0	0	0	0	0	0	0	1, 4	B
	+	+	0	0	0	0	0	0	3	
	+	+	+	+	+	0	0	0	2	
16	0	0	0	0	0	0	0	0	2	C
	+	+	0	0	0	0	0	0	4	
	+	+	+	0	0	0	0	0	3	
	+	+	+	+	0	0	0	0	1	
13	0	0	0	0	0	0	0	0	1	D
	+	+	+	0	0	0	0	0	3, 4	
	+	+	+	+	0	0	0	0	2	

# *Reproduction of Aerobic Bacteria*

## CHART 3, GROUP G—*contd.*

### MORPHOLOGY OF LISTER B. FLEXNER CULTURE (STOCK).

Dominant population "normal" bacilli.

### MORPHOLOGY OF B. FLEXNER SUBCULTURES.

1. Slender bacilli, and many bacilli showing Artaud's nodes.
2. "Normal" bacilli.
3. Short fat bacilli, uniformly stained.
4. Small coccoids, large diplococcoids, large ovoids.

### MORPHOLOGY OF LISTER B. Y CULTURE (STOCK).

Dominant population very minute cocco bacilli.

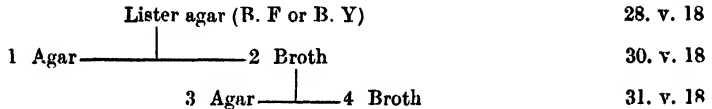
### MORPHOLOGY OF B. Y SUBCULTURES.

1. Minute coccoids and diplococcoids.
2. ? "Normal" bacilli.
3. Minute coccoids and minute bacilli.
4. Minute coccoids and diplococcoids : no bacilli.

## *B. dysenteriae.*

### KEY TO CHART 3, GROUP G.

Key to Cultures:



Key to Agglutinations:

- A. Lister B. Flexner Culture v. Lister B. Flexner serum C 94 : 1/20 : 1/3000 : 27. III. 18
- B. Lister B. Flexner Culture v. Lister B. Y serum C 137 : 1/10 : 1/1500 : 27. III. 18.
- C. Lister B. Y Culture v. Lister B. Flexner serum C 94 : 1/20 : 1/3000 : 27. III. 18.
- D. Lister B. Y Culture v. Lister B. Y serum C 137 : 1/10 : 1/1500 : 27. III. 18.

## CHART 3, GROUP H.

*B. dysenteriae*: R.A.M.C. B. Flexner Stock Culture and R.A.M.C. B. Y Stock Culture *versus* R.A.M.C. Serums B. Flexner and B. Y.

E.A.M.	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control	Cultures	Experiment
15	+	+	0	0	0	0	0	0	2	A
	+	+	+	+	0	0	0	0	5, 6, 4	
	+	+	+	+	+	+	0	0	1, 3	
10	+	+	0	0	0	0	0	0	4	B
	+	+	+	0	0	0	0	0	5, 6	
	+	+	+	+	0	0	0	0	1, 2, 3	
6	0	0	0	0	0	0	0	0	2, 5, 6, 4, 3	C
	+	+	+	+	0	0	0	0	1	
17	+	+	0	0	0	0	0	0	6	D
	+	+	+	0	0	0	0	0	3	
	+	+	+	+	0	0	0	0	2, 4	
	+	+	+	+	+	0	0	0	1, 5	

CHART 3, GROUP H—*contd.*

## MORPHOLOGY OF B. FLEXNER CULTURES 3 TO 6.

3. Short bacilli.
4. "Normal" bacilli.
5. Coccoids and bacilli.
6. Short bacilli.

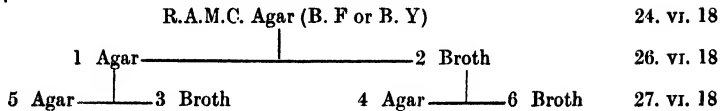
## MORPHOLOGY OF B. Y CULTURES 3 TO 6.

3. Bacilli greatly varying in size: large spheroidal forms.
4. Large bacilli: numerous minute bacteroids.
5. ? "Normal" bacilli.
6. Large broad bacillary forms.

*B. dysenteriae.*

## KEY TO CHART 3, GROUP H.

## Key to Cultures:



## Key to Agglutinations:

- A. R.A.M.C. B. Flexner Culture Ledingham 24. vi. 18 v. R.A.M.C. Flexner Ledingham serum F 7 : 1/20 : 1/8000 : 27. vi. 18.
- B. R.A.M.C. B. Flexner Culture Ledingham 24. vi. 18 v. R.A.M.C. Y Ledingham serum : 1/7000 : 27. vi. 18.
- C. R.A.M.C. Y Culture Ledingham 24. vi. 18 v. R.A.M.C. Flexner Ledingham serum F 7 : 1/20 : 1/8000 : 27. vi. 18.
- D. R.A.M.C. Y Culture Ledingham 24. vi. 18 v. R.A.M.C. Y Ledingham serum : 1/7000 : 27. vi. 18.

## CHART 3, GROUP I.

*B. dysenteriae*: R.A.M.C. B. Flexner Culture Ledingham *versus* R.A.M.C.  
B. Flexner, Lister B. Flexner and Lister B. FY Serums.

E.A.M.	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Cultures	Serums	Exp.
15	+	+	0	0	0	0	0	0	0	2	R.A.M.C.	A
	+	+	+	+	0	0	0	0	0	5, 6, 4		
	+	+	+	+	+	+	0	0	0	1, 3	culture	24. vi. 18
42	+	+	0	0	0	0	0	0	0	4	R.A.M.C.	B
	+	+	+	+	+	+	0	0	0	5		
	+	+	+	+	+	+	+	+	0	1, 3	culture	2. x. 18
	+	+	+	+	+	+	+	+	+	2, 6		
5	0	0	0	0	0	0	0	0	0	1, 4, 5	Lister F C 94	
	+	+	0	0	0	0	0	0	0	3, 6		
	+	+	+	0	0	0	0	0	0	2		
32	+	+	0	0	0	0	0	0	0	4	Lister F Y C 70	
	+	+	+	+	0	0	0	0	0	5		
	+	+	+	+	+	0	0	0	0	1		
	+	+	+	+	+	+	...	...	...	3, 6		
	+	+	+	+	+	+	+	0	0	2		



Reproduction of Aerobic Bacteria

CHART 3, GROUP I—*contd.*

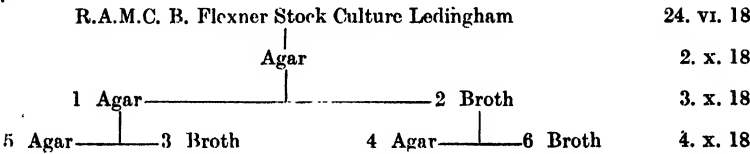
MAXIMUM VARIATIONS OBTAINED IRRESPECTIVE OF SERUMS EMPLOYED.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480
0	...	...	...	...	...	...	...	...
+	+	...	...	...	...	...	...	...
+	+	+	...	...	...	...	...	...
+	+	+	+	...	...	...	...	...
+	+	+	+	+	...	...	...	...
+	+	+	+	+	+	...	...	...
+	+	+	+	+	+	+	+	...
+	+	+	+	+	+	+	+	+

*B. dysenteriae.*

KEY TO CHART 3, GROUP I.

Key to Cultures:



Key to Agglutinations:

- A. R.A.M.C. B. Flexner Culture Ledingham 24. vi. 18 *v.* R.A.M.C. B. Flexner Ledingham serum F 7: 1/20: 27. vi. 18.
- B. R.A.M.C. B. Flexner Culture Ledingham 2. x. 18 *v.* R.A.M.C. B. Flexner Ledingham serum F 7: 1/20: 27. vi. 18.
- C. R.A.M.C. B. Flexner Culture Ledingham 2. x. 18 *v.* Lister B. Flexner serum C 94: 1/20: 1/3000: 27. iii. 18.
- D. R.A.M.C. B. Flexner Culture Ledingham 2. x. 18 *v.* Lister B. FY serum C 70: 1/10: 1/2000: 12. i. 18(?).

CHART 3, GROUP K 1.

*B. dysenteriae:* R.A.M.C. B. Flexner Culture Ledingham *versus* R.A.M.C. Serums FY 3, Y 55, F 8 A and Lister Serum FY C 137.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Plate	Medium
+	+	0	0	0	0	0	0	0	A	G $\frac{1}{2}$ colony	Agar
+	+	+	0	0	0	0	0	0	B, C	"	"
+	+	+	+	+	+	+	+	+	D	"	"
+	+	+	+	0	0	0	0	0	B	G $\frac{1}{2}$ colony	Broth
+	+	+	+	+	+	0	0	0	C	"	"
+	+	+	+	+	+	+	0	0	A	"	"
+	+	+	+	+	+	+	+	+	D	"	"
+	0	0	0	0	0	0	0	0	B, D	H $\frac{1}{2}$ colony	Agar
+	+	+	0	0	0	0	0	0	A, C	"	"
+	+	+	+	0	0	0	0	0	A, B	H $\frac{1}{2}$ colony	Broth
+	+	+	+	+	0	0	0	0	C	"	"
+	+	+	+	+	+	+	0	0	D	"	"

(A) R.A.M.C. serum F Y 3; (B) R.A.M.C. serum Y 55; (C) Lister serum FY C 137; (D) R.A.M.C. serum F 8 A.

CHART 3, GROUP K 1—*contd.*

### MAXIMUM VARIATION IRRESPECTIVE OF SERUM.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240
+	...	...	...	...	...	...	...
+	+	...	...	...	...	...	...
+	+	+	...	...	...	...	...
+	+	+	+	...	...	...	...
+	+	+	+	+	...	...	...
+	+	+	+	+	+	+	...
+	+	+	+	+	+	+	...
+	+	+	+	+	+	+	+

CHART 3, GROUP K 2.

*B. dysenteriae*: R.A.M.C. B. Flexner Culture Ledingham *versus* R.A.M.C. Serums FY 3, Y 55, F 8 A and Lister Serum C 94.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Plate	Medium
0	0	0	0	0	0	0	0	0	A	D $\frac{1}{2}$ colony	Agar
0	0	0	0	0	0	0	0	0	A	D $\frac{1}{2}$ "	Broth
0	0	0	0	0	0	0	0	0	A	C $\frac{1}{2}$ "	Agar
+	+	0	0	0	0	0	0	0	A	C $\frac{1}{2}$ "	Broth
+	+	+	0	0	0	0	0	0	B	D $\frac{1}{2}$ "	Agar
+	+	+	0	0	0	0	0	0	B	C $\frac{1}{2}$ "	Agar
+	+	+	+	0	0	0	0	0	B	D $\frac{1}{2}$ "	Broth
+	+	+	+	0	0	0	0	0	B	C $\frac{1}{2}$ "	Broth
0	0	0	0	0	0	0	0	0	C	D $\frac{1}{2}$ "	Broth
+	+	+	0	0	0	0	0	0	C	D $\frac{1}{2}$ "	Agar
+	+	+	0	0	0	0	0	0	C	C $\frac{1}{2}$ "	Agar
+	+	+	+	+	0	0	0	0	C	C $\frac{1}{2}$ "	Broth
+	+	+	+	0	0	0	0	0	D	D $\frac{1}{2}$ "	Agar
+	+	+	+	+	+	+	+	0	D	D $\frac{1}{2}$ "	Broth
+	+	+	+	+	+	+	+	0	D	C $\frac{1}{2}$ "	Agar
+	+	+	+	+	+	+	+	0	D	C $\frac{1}{2}$ "	Broth

(A) Lister B. Flexner serum C 94; (B) R.A.M.C. serum Y 55; (C) R.A.M.C. serum FY 3;  
(D) R.A.M.C. serum F 8 A.

### MAXIMUM VARIATION IRRESPECTIVE OF SERUM.

[illegible]

*Reproduction of Aerobic Bacteria*

## CHART 3, GROUP K 3.

*B. dysenteriae*: R.A.M.C. B. Flexner Culture Ledingham *versus* R.A.M.C. Serums FY 3, Y 55, F 8 A and Lister Serum C 94.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Plate	Medium
0	0	0	0	0	0	0	0	0	A	E $\frac{1}{2}$ colony	Agar
+	+	0	0	0	0	0	0	0	B	"	"
+	+	+	0	0	0	0	0	0	C	"	"
+	+	+	+	+	+	+	+	+	D	"	"
+	0	0	0	0	0	0	0	0	D	E $\frac{1}{2}$ colony	Broth
+	+	+	0	0	0	0	0	0	B	"	"
+	+	+	+	0	0	0	0	0	C	"	"
+	+	+	+	+	0	0	0	0	A	"	"
+	0	0	0	0	0	0	0	0	A	F $\frac{1}{2}$ colony	Agar
+	+	+	0	0	0	0	0	0	C	"	"
+	+	+	+	0	0	0	0	0	B	"	"
+	+	+	+	+	+	+	+	+	D	"	"
+	0	0	0	0	0	0	0	0	A	F $\frac{1}{2}$ colony	Broth
+	+	+	0	0	0	0	0	0	B	"	"
+	+	+	+	+	0	0	0	0	C	"	"
+	+	+	+	+	+	+	+	+	D	"	"

(A) Lister B. Flexner serum C 94; (B) R.A.M.C. serum B. Y 55; (C) R.A.M.C. serum B. FY 3; (D) R.A.M.C. serum F 8 A.

## MAXIMUM VARIATION IRRISPCTIVE OF SERUM.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480
+	...	...	...	...	...	...	...	...
+	+	...	...	...	...	...	...	...
+	+	+	...	...	...	...	...	...
+	+	+	+	...	...	...	...	...
+	+	+	+	+	...	...	...	...
+	+	+	+	+	+	+	+	+

## CHART 4, GROUP L 1.

*B. dysenteriae*: B. Y Kitchener Strain (B) *versus* R.A.M.C. Polyvalent Serum, FY 3, Y 55, Y 4, and Lister FY C 137 Serum.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Cultures
+	+	0	0	0	0	0	0	0	R.A.M.C. FY 3	18, 19
+	+	+	0	0	0	0	0	0		12
+	+	+	+	0	0	0	0	0		10, 13
+	+	+	+	+	0	0	0	0		11
+	+	+	0	0	0	0	0	0	Lister FY C 137	8, 9, 12
+	+	+	+	0	0	0	0	0		10
+	+	+	+	+	0	0	0	0		11
+	+	+	+	+	+	0	0	0		13
+	+	0	0	0	0	0	0	0	R.A.M.C. Polyvalent	20, 21
+	+	+	+	+	0	0	0	0		14
+	+	+	+	+	+	0	0	0		16
+	+	+	+	+	+	0	0	0		15
+	+	+	+	+	+	0	0	0		17

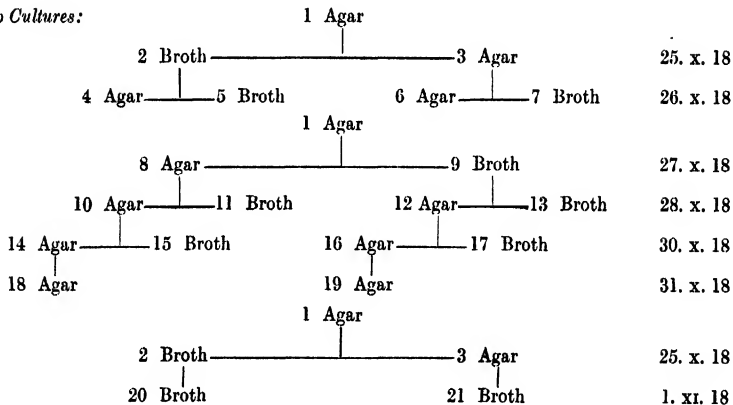
CHART 4, GROUP L 1—*contd.*

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	Serum	Cultures
+	+	+	0	0	0	0	0	0	R.A.M.C. Y 55	13
+	+	+	+	+	0	0	0	0		8, 9
+	+	+	+	+	+	0	0	0		1, 7, 12
+	+	+	+	+	+	+	0	0		3, 6
+	+	+	+	+	+	+	+	...		10, 11
+	+	+	+	+	+	+	+	+	R.A.M.C. Y 4	2, 4, 5
+	+	+	0	0	0	0	0	0		20, 21

## MAXIMUM VARIATIONS IRRESPECTIVE OF SERUMS.

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480
+	+	...	...	...	...	...	...	...
+	+	+	...	...	...	...	...	...
+	+	+	+	...	...	...	...	...
+	+	+	+	+	...	...	...	...
+	+	+	+	+	+	...	...	...
+	+	+	+	+	+	+	...	...
+	+	+	+	+	+	+	+	...
+	+	+	+	+	+	+	+	+

Key to Cultures:



*B. dysenteriae*: *B. Shiga*-Kruse Culture Oxford versus Oxford Shiga Serum,  
R.A.M.C. Shiga Serum and Lister Shiga Serum.

<b>1/80</b>	<b>1/160</b>	<b>1/320</b>	<b>1/640</b>	<b>1/1280</b>	<b>1/2560</b>	<b>1/5120</b>	<b>1/10,240</b>	<b>1/20,480</b>	<b>1/40,960</b>	<b>Scrum : cultures</b>
										<b>R.A.M.C.</b>
+	...	...	...	...	...	...	...	...	...	<b>25, 27, 28</b>
+	+	+	+	...	...	...	...	...	...	<b>19</b>
+	+	+	+	+	...	...	...	...	...	<b>14</b>
+	+	+	+	+	+	...	...	...	...	<b>13, 21</b>
+	+	+	+	+	+	+	...	...	...	<b>20, 31, 32</b>
+	+	+	+	+	+	+	+	+	...	<b>15</b>
										<b>Oxford</b>
+	...	...	...	...	...	...	...	...	...	<b>19, 20, 21, 25</b>
+	+	+	+	+	+	...	...	...	...	<b>28</b>
+	+	+	+	+	+	+	+	+	...	<b>31, 32</b>
+	+	+	+	+	+	+	+	+	+	<b>13, 14, 15, 27</b>
										<b>Lister</b>
+	...	...	...	...	...	...	...	...	...	<b>14</b>
+	+	...	...	...	...	...	...	...	...	<b>13</b>
+	+	+	...	...	...	...	...	...	...	<b>15</b>
+	+	+	+	+	+	+	+	...	...	<b>25, 28</b>
+	+	+	+	+	+	+	+	+	...	<b>27</b>

„	R.A.M.C.	„	4	„	11	„	„	„
„	Lister	„	2	„	6	„	„	„

[illegible]

CHART 5, GROUP M—*contd.*

1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10,240	1/20,480	1/40,960	Cultures	Serum
+	+	+	+	+	+	+	+	0	0	25	Lister
+	+	+	+	+	+	+	+	0	0	28	
+	+	+	+	+	+	+	+	+	0	27	„
+	+	+	+	+	+	+	0	0	0	31	R.A.M.C
+	+	+	+	+	+	+	0	0	0	32	„
+	+	+	+	+	+	+	+	+	0	31	Oxford
+	+	+	+	+	+	+	+	+	0	32	„

Key to Cultures:

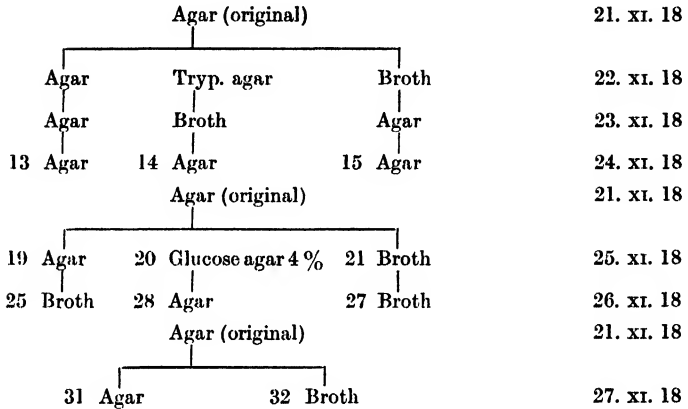


CHART 6, GROUP N.

Meningococcus Type 1 *versus* R.A.M.C. Serums Types 1, 2, 3, 4.

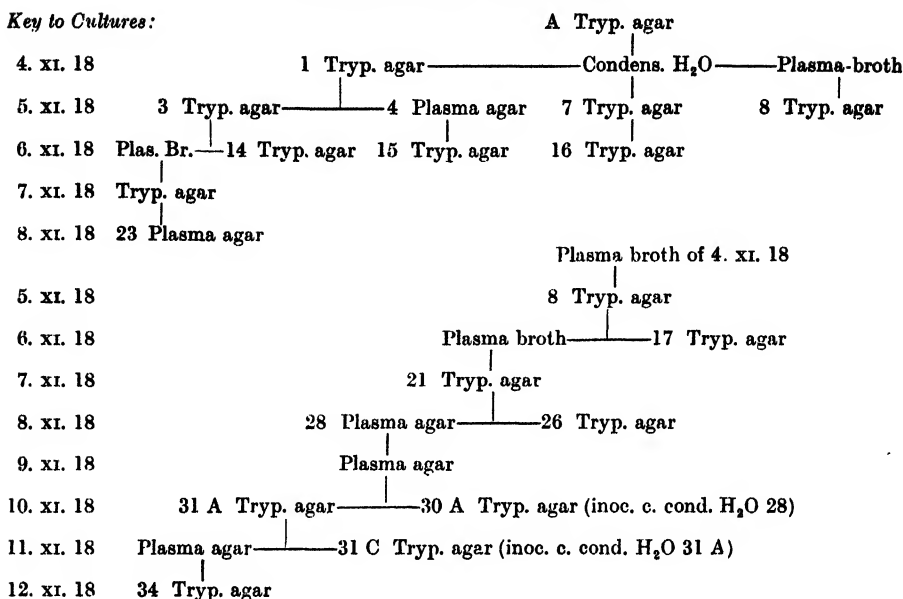
0	1/100	1/200	1/400	1/800	1/1600	Control	Cultures	Serums	Serums
+	0	0	0	0	0	0	14, 21	(J 4 E.T. 1/1000)	Type 4
+	+	0	0	0	0	0	1, 15, 16, 17, 30 A	—	—
+	+	+	0	0	0	0	3, 7, 8, 26, 31 A, 31 C, 34	—	—
+	+	+	+	0	0	0	23	—	—
+	+	+	+	+	0	0	4	—	—
+	0	0	0	0	0	0	1, 3, 4, 21	(J 3 E.T. 1/800)	Type 3
+	+	0	0	0	0	0	30 A, 34	—	—
+	+	+	0	0	0	0	7, 8, 14, 15, 26, 31 A	—	—
+	+	+	+	0	0	0	16, 17, 31 C	—	—
+	+	+	+	+	0	0	23	—	—
+	+	0	0	0	0	0	1, 3, 4, 23, 26, 30 A	(N 2 E.T. 1/800)	Type 2
+	+	+	0	0	0	0	8, 14, 15, 17, 21, 31 A, 34	—	—
+	+	+	+	0	0	0	7, 16	—	—
+	+	+	+	+	0	0	31 C	—	—
+	+	+	0	0	0	0	34, 31 C	(F 1 E.T. 1/1200)	Type 1
+	+	+	+	0	0	0	4, 7, 16, 21, 26, 30 A, 1	—	—
+	+	+	+	+	0	0	3, 8, 14, 15, 17, 23, 31 A	—	—
E.A.M. with serum type 4							17-2		
„ „ „							3	17-10	
„ „ „							2	19-12	
„ „ „							1	29-12	

Cultures 4, 23, 31 C agglutinate with serums 2, 3 or 4 respectively as well as does any single culture with serum 1: 1/800.

Cultures 23, 16, 17, 31 C, 7 agglutinate with serums 2, 3 or 4 respectively as well as do cultures 4, 7, 16, 21, 26, 30 A, 1 with serum 1: 1/400.

CHART 6, GROUP N—*contd.*

## Key to Cultures:

*Meningococcus Type 1: Morphological Equivalents of Subcultures.*

A. Chiefly small equally dividing meningococci: several deeply staining "giant" forms, some dividing equally, others unequally (budding), others showing central clear space.

1. Normal meningococci, equally dividing: uniform in size and staining: no "giant" forms seen.

3. Large non-dividing forms: three and four times the diameter of meningococci.

4. Normal meningococci mixed with minute forms, some of which are about 0.2 microns in diameter: a few small deeply-staining non-dividing forms.

7. Normal dividing meningococci, some very minute: no "giant" forms seen.

8. Normal dividing meningococci, some very minute: a few "giant" forms, numerous Gram negative minute bacilli: organisms stain uniformly.

14. *Vide* 15.

15. Medium sized "giant" forms, only a few showing signs of division: several small bacillary forms (Arkwright, d'Este Emery and others).

16. Dominant population lightly-staining "giant" forms, many undergoing unequal fission: occasional small bacillary forms.

23. Large organisms staining uniformly: many staining deeply with double contour outline, suggesting thick walls.

21. Large deeply-staining organisms, occurring singly, in short chains and in clusters. No evidence of division. Some have clear centres, in others the clear space is placed laterally, perhaps a profile effect. Population as a whole homogeneous in size. In some cases the clear centre occupies the greater part of the cell. The film does not in the least degree represent a meningococcal preparation.

26. Normal dividing meningococci, mixed in some parts of the film with large numbers of small bacilli, which are in many cases throwing off Gram negative buds, indistinguishable from meningococci: several bacilli are undergoing spherical segmentation in the terminal part of the bacillary axis.

17. No meningococci seen: population appearing entirely to consist of "giant" cells of all sizes, mainly undergoing unequal binary fission.

31 A. "Giant" cells of all sizes, from very small to large: some staining deeply, with thickened walls. A few equally dividing meningococci seen, and a few minute bacillary forms, some being of the wisp type.

30 A. Normal equally dividing meningococci, and a few large "giant" forms, with deeply-staining outline, and clear centres: numerous chains of 3, 4, 5 or 6 organisms: one chain of 8 organisms.

34. Extremely minute single and dividing cocci, some measuring about 0.1 to 0.2 microns in diameter. Some of the larger forms, from 0.4 upwards, stain more deeply than the smaller forms, some of which occur in short chains.

(Numbers refer to films and not to cultures.)

#### ROUGH SUMMARY OF RESULTS.

Although true meningococci are represented in serum 1, as shown by the high agglutination figure reached with serum 1 in the case of subculture 8, it appears that the antigenic value of strain 1, as represented in serum 1, finds its chief expression in "giant" forms, the morphological equivalent of films 3, 14, 17, 23, 31 A, from the subcultures which give the highest readings with serum 1 (except 8), being mainly that of "giant" forms in various stages of development. As I showed in 1916 "giant" forms may be either large, small or intermediate in size, and can at once be distinguished from true meningococci on the warm-stage by the fact that they often multiply by unequal binary fission, and by the further fact that they often undergo in addition endo-chromatinolysis, giving rise directly to meningococci which then divide in the ordinary vegetative manner. These fertile "giant" forms are readily distinguishable from forms undergoing genuine involutionary changes, both by the sterility of the latter when observed on the warm stage and by their feebly-staining properties.

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## EXPLANATION OF PLATES IV—VII.

## PLATE IV.

*B. typhosus* subcultured on + 10 agar from 4 % glucose broth. Composite selected fields. Agar cultures 1 to 3 hours, glucose cultures 12 hours.

1. Thick-walled cells (a) resting, (b) germinating. 2. Bacteroids living. 3. Sporangoids living. 4. Chlamydosporoids living. 5. Oidioids living. 6. Gonidioids living. 7. Coccoids stained. 8. Giant-cells stained. 9. Flagella unstained. 10. Mixed field living.

Series 1—10,  $\times 1500$ .

## PLATE V.

*B. typhosus*: warm-stage observations on + 10 agar from 4 % glucose broth.

Series A—D,  $\times 1500$ .

## PLATE VI.

*B. typhosus*: warm-stage observations on + 10 agar from 4 % glucose broth.

Series E—J,  $\times 3000$ .

## PLATE VII.

*B. typhosus*. Developmental phases associated with loss of agglutinability (photographs by F. Martin Duncan,  $\times 1000$ ).

- 1, 5. Thin-walled coccoids (agglutination nil), Lister serum.
- 4, 8. Thick-walled coccoids and normal bacilli (agglutination 1/1280), Lister serum.
- 2, 3, 6. Thick-walled coccoids (agglutination nil), Lister serum.
7. Thick-walled coccoids and bacilli (agglutination 1/160), Lister serum.

Pure bacillary subcultures from these coccoids agglutinated to 1/40,960, Lister serum. For warm-stage observations of growth from coccoid to bacillus *v.* Plate VI, G.

## TIME-TABLE OF PLATES V AND VI.

- A. 4.55, 5.10, 5.22, 5.35, 5.45, 6.0, 6.12, 6.50 p.m.
- B. 7.35, 7.50, 8.15, 8.40, 9.0, 9.20, 9.40, 10.0 p.m.
- C. 8.40, 9.10, 9.40, 10.10, 10.30 p.m.
- D. 7.0, 7.40, 8.10, 9.0, 9.15, 9.50, 10.15 p.m.
- E, F. 10.0, 10.5, 10.10, 10.20, 10.25, 10.40, 11.10 to 12.0 p.m.
  1. 7.30, 8.0, 8.35, 9.0 p.m.
  - J. 6.25, 6.35, 6.44, 6.48 p.m.

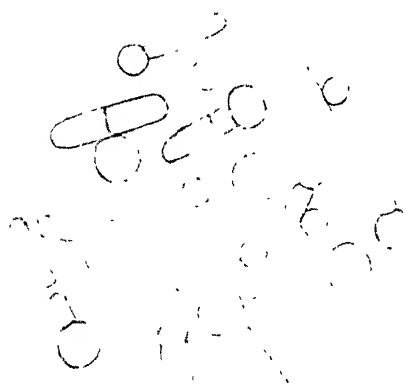




1



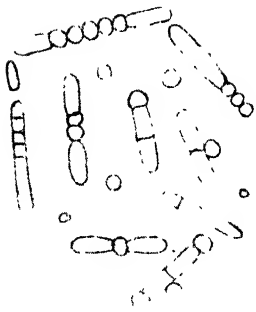
2



3



4



5



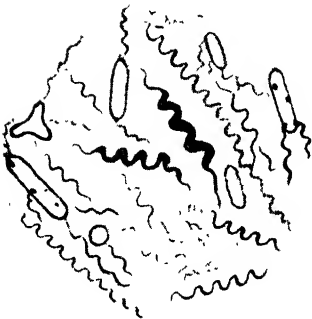
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7



8

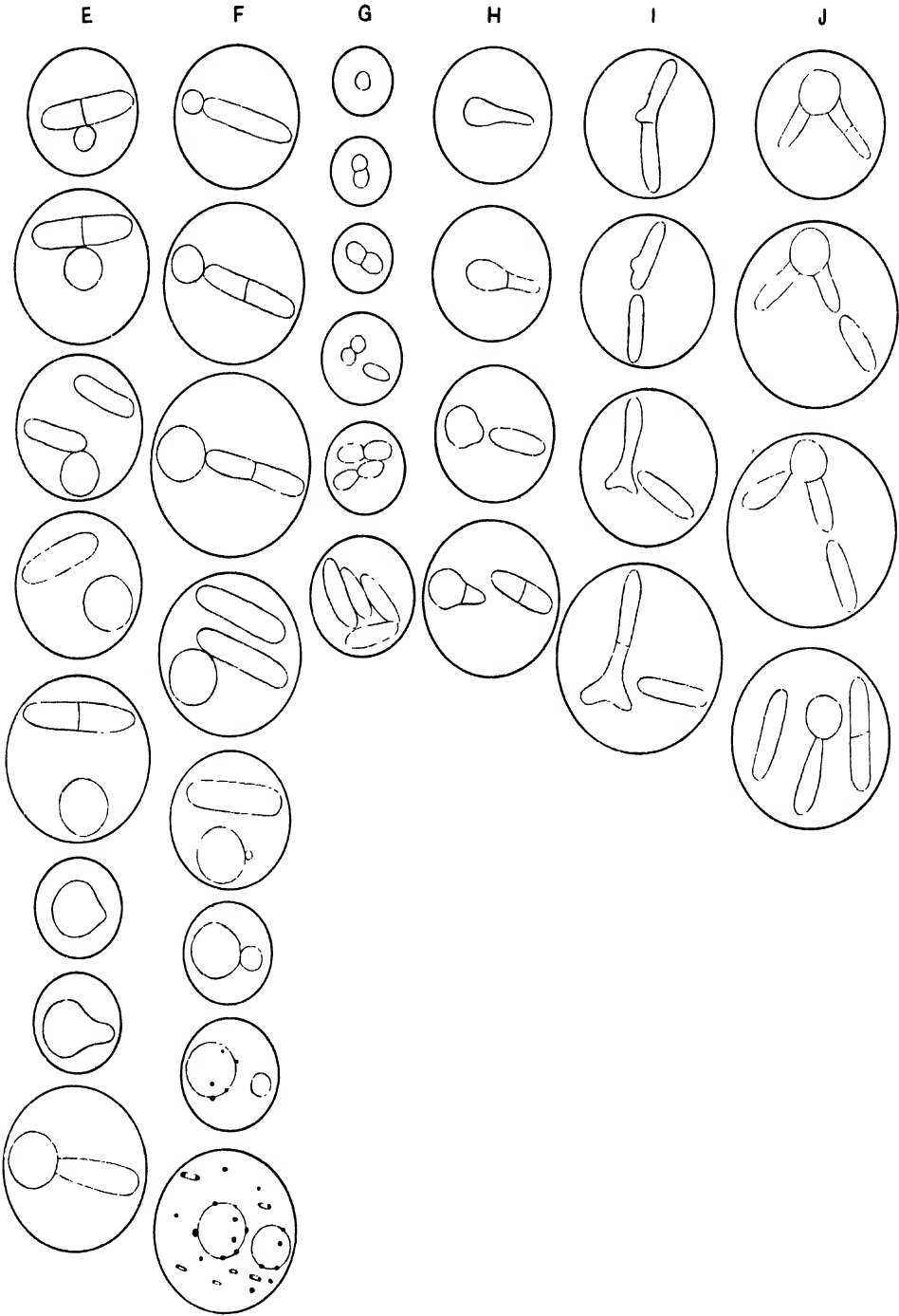


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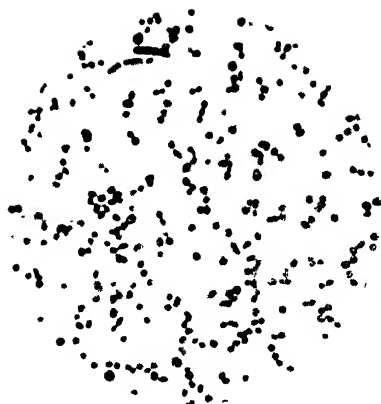
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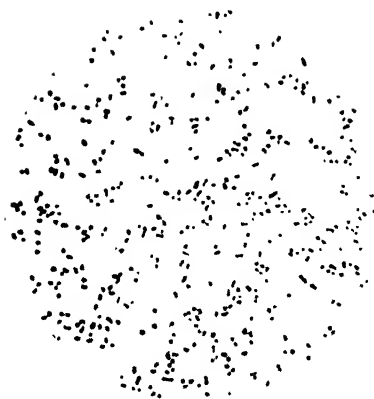




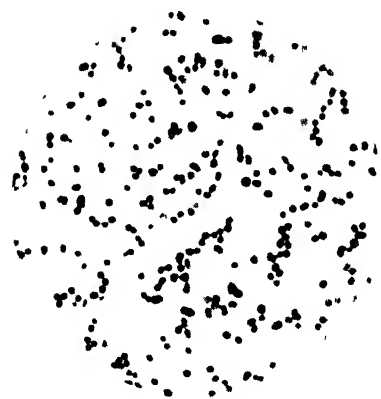
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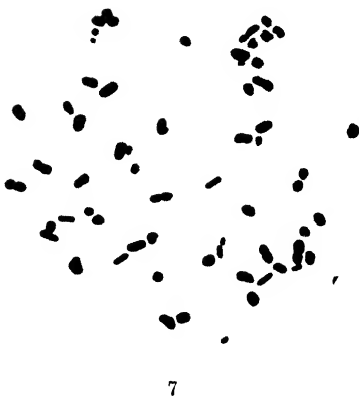
2



5



6







## THE DIETS OF LABOURING CLASS FAMILIES DURING THE COURSE OF THE WAR.

By MARGARET FERGUSON, M.A.

(*From the Physiological Laboratory, University of Glasgow.*)

IN 1915-1916 I studied the dietaries of forty-seven Glasgow labouring class families with the object of comparing the food of healthy and rachitic children. The results of this work were published by the Medical Research Committee<sup>1</sup>.

In order to ascertain how the food was affected by war conditions subsequent studies were made of a few typical families at three different periods.

(1) Ten families were investigated in February 1917, when, though prices were high, all food-stuffs, save potatoes, were still plentiful.

(2) Eight of these families consented to a third study, which was carried out in November 1917, after voluntary rationing had been urged by the Minister of Food.

(3) In December 1918 five of these families were studied for a fourth time, this time during the period of compulsory rationing of meat, sugar and fats.

We have, thus, a series of four investigations of the same five families under different conditions and extending over a period of three years of the war. The present report summarises the information gathered.

The studies included three men, five women, twelve children over ten years and fourteen under ten years of age.

In each case the food was weighed for the period of a week.

The method of investigation adopted is explained in Professor Noël Paton's introduction to the report of the dietary studies made by Miss Dorothy Lindsay in 1911-1912. Miss Lindsay's work was done in Glasgow upon the same class, and forms a pre-war standard of comparison with the present results.

A comparison of the results is given in Table I.

### I. *The effect of Rationing.*

The present study shows that rationing had little effect upon the protein content or the energy value of these diets, but that the average consumption of fat fell 14 gms. per man per day. Four of the five families consumed less fat. Where strictest economy is necessary, as was the case here, the housewife generally relies on margarine as her chief source of fat, the fats in meat being

<sup>1</sup> See *Special Report Series*, No. 20, 1918, and also, from the economic standpoint, in *Proc. Roy. Soc. Edinburgh*, Vol. xxxvii, part II.

so much more expensive. Dripping and lard could have been used instead of margarine, but that would have been contrary to the usual dietary habits of the families in question, and the housewives seem to have preferred simply to reduce the allowance of fat. Table II shows the extent of the reduction.

Table I.

*Energy Value, Protein and Fat Consumption per man per day calculated on the basis of Atwater's allowances.*

	First study, 1915-16				Second study, Feb. 1917			
	Protein in gms.	Fat in gms.	Energy in calories	Family income	Protein in gms.	Fat in gms.	Energy in calories	Family income
S 84	86.0	93.0	2836	27s.	77.5	63.8	2530	30s.
N 31	128.9	128.2	4174	36s.	103.0	67.8	3112	39s.
H 47	88.9	67.2	3003	22s.	85.0	62.3	2714	23s. 5d.
M 112	88.0	97.5	3318	30s.	105.9	98.1	3476	55s. 6d.
N 150	148.4	105.3	3568	25s.	138.6	112.8	3690	35s. 6d.
Average	108.0	98.2	3380	—	102.0	81.0	3104	—
Average*	93.1	84.2	2897	—	87.4	69.4	2661	—
	Third study, Nov. 1917				Fourth study, Dec. 1918			
	Protein in gms.	Fat in gms.	Energy in calories	Family income	Protein in gms.	Fat in gms.	Energy in calories	Family income
S 84	82.3	66.0	2289	38s.	79.9	65.3	2713	55s.
N 31	123.1	77.2	4079	44s.	104.7	60.3	2892	41s. 6d.
H 47	100.1	77.2	3159	34s.	93.3	62.9	3003.1	34s.
M 112	119.9	134.6	3650	41s. 61s.	100.0	71.0	3332.1	61s.
N 150	105.0	92.7	3202	48s.	146.1	117.4	3691.1	68s.
Average	106.1	89.5	3276	—	104.8	75.4	3126	—
Average*	87.5	76.7	2808	—	89.6	64.6	2680	—

\* Corrected to Lusk standard (p. 411).

Table II.

*Amounts consumed per man per week in lbs. on the basis of Atwater's allowances, and the food value purchased per penny spent in each study.*

	First study							Second study						
	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per lb.	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per lb.
S 84	4.97	4.03	1.0	.86	2267	.51	354	5.64	.78	.91	.84	739	.64	265
N 31	7.51	1.7	1.69	2.08	2191	.52	413	7.58	.7	1.6	.68	801	.45	324
H 47	4.0	4.0	.91	1.52	868	.50	418	7.69	—	.62	.57	340	.57	330
M 112	4.55	4.52	1.71	1.43	2079	.71	400	7.87	2.1	1.79	.87	808	.85	303
N 150	7.04	8.16	3.69	.56	2464	.21	277	7.08	5.55	3.29	1.23	1360	.49	182
Av.	5.61	4.48	1.8	1.29	1974	.49	372	7.17	1.83	1.74	.84	810	.6	281
	Third study							Fourth study						
	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per lb.	Flour	Potatoes	Meat	Sugar	Milk in c.c.s	Butter and margarine	Calories per lb.
S 84	6.26	2.04	1.18	.52	1041	.33	222	4.69	2.93	1.15	1.25	1420	.47	217
N 31	9.63	5.12	.97	1.16	2431	.88	378	7.55	3.49	1.74	.81	1453	.47	223
H 47	7.06	5.95	.96	.58	800	.57	345	6.89	3.68	.99	.83	477	.46	269
M 112	6.32	5.72	1.21	.90	1737	1.32	241	7.72	3.38	2.12	1.34	1560	.32	192
N 150	5.13	9.9	2.72	.73	1459	.58	203	6.10	5.40	4.0	.50	2470	.20	148
Av.	6.88	5.75	1.41	.78	1494	.74	278	6.59	3.78	2.0	.95	1476	.38	210

The diet which shows an increase of fat (N 150) is that of a family, which had a considerable advance of income during the war. The fat eaten by this family came mainly from meat, particularly from unrationed sausages and bacon. Indeed they did not draw their full margarine ration, and the mother was in the habit of using a small quantity of dripping each day to light the fire!

The official weekly rations at that time were: Sugar  $\frac{1}{2}$  lb., Margarine  $\frac{1}{4}$  lb. and Butter 1 oz. Meat (excluding rabbits, bacon and sausages, which were not rationed) could be purchased to the value of 1s. 3d. per person per week. There were also lard and jam rations each amounting to  $\frac{1}{4}$  lb. weekly. The former none, and the latter only two of the families purchased.

*Meat.* All the families had slightly more meat at the fourth than during previous studies. This was probably due to the butter and margarine restrictions. The average amount of rationed meat eaten during the study week was only  $\frac{1}{2}$  lb. per person.

*Sugar.* Families S 84 and M 112 had the Christmas increase to  $\frac{3}{4}$  lb. per person. N 150, on the other hand, did not use the full  $\frac{1}{2}$  lb. ration. On the whole the sugar consumed was more than at the previous study, though not so much as in 1915.

Rationing thus brought about singularly little change. The more determinative factors were income, and the dietary habits of the families.

## II. *A Consideration of the Adequacy of the Diets.*

From Table I it will be seen that, with one exception, there is a singular uniformity in the energy value of the diet of each family at the four different periods. This, however, is no guarantee that the food is sufficient. Recent investigations would seem to show that Atwater's allowances for age and sex, upon which these and all previous dietary studies have been calculated, are not adequate. Lusk has suggested that the following values may be taken as representing the ratio of the food requirements of the child to that of the average man, and this has been accepted by the Food Committee of the Royal Society, and the International Committee.

LUSK		ATWATER	
Age	Coefficients	Age	Coefficients
0- 6	0.5	Under 2	0.3
6-10	0.6	2- 5	0.4
10-13	0.83	6- 9	0.5
13-20 (boys)	1.0	10-13	0.6
Average man	1.0	14-16 (boys)	0.8
		Average man	1.0
13-20 (girls)	0.83	14-16 (girls)	0.7
Average woman	0.83	Average woman	0.8

These figures were calculated on the basis of the standard measurements of the Anthropometric Committee, 1883, and on the experimental work of Du Bois, who proved that the energy expended per unit of body surface is greater

*Diets of Labouring Class*

in children than in the adult male. The adoption of Lusk's coefficients instead of Atwater's raises the average equivalent per person in the five families from 0.6 to 0.7 of a man. The values of the diets according to Lusk's allowances are given at the foot of Table I. These are much below what is usually thought necessary. The explanation of this probably lies in the fact that in three of the families the children were markedly below the Anthropometric Committee's averages. Omitting N 150, where there were only two small children under five years, whose dietary needs were small compared with those of the parents, the figures were as follows:

*Height in cms.*

Age	Anthropometric Committee		N 31		S 84		H 47		M 112	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
2	85.5	81.1	—	—	—	—	66.7	—	—	—
5	104.2	103	—	—	91.4	—	—	—	—	—
6	111.8	108.9	—	—	—	—	90.1	—	—	—
7	116.8	112.9	—	—	101.0	—	—	—	108.6	—
8	119.5	118.4	—	—	—	—	—	105.4	—	—
9	126.2	123.8	126	—	—	125	—	—	111.8	—
10	131.7	129.7	132	—	—	—	—	107.6	—	—
11	135.8	134.9	—	—	131.4	—	—	—	—	—
12	139.7	141.4	—	—	—	—	123.2	—	—	125.7
13	144.6	146.7	—	144	—	—	—	125.7	—	125.7
14	150.7	151.9	—	153	138.4	—	—	—	136.5	—
15	155.4	154.6	—	156	—	—	—	—	—	—
Adult	171	159.3	—	159.2	174	161.3	170.1	156.2	—	154.7

*Weight in kgms. (without clothing).*

Age	Anthropometric Committee		N 31		S 84		H 47		M 112	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
5	18.1	17.8	—	—	14.32	—	—	—	—	—
6	20.1	18.9	—	—	—	—	14.0	—	—	—
7	22.5	21.6	—	—	15.91	—	—	—	17.27	—
8	24.9	23.6	—	—	—	—	—	19.32	—	—
9	27.4	25.2	22.05	—	—	20.45	—	—	20.0	—
10	30.6	28.1	25.45	—	—	—	—	19.54	—	—
11	32.7	30.8	—	—	30.5	—	—	—	—	—
12	34.8	34.6	—	—	—	—	25.0	—	—	23.87
13	37.5	39.5	—	31.82	—	—	—	26.82	—	23.4
14	41.7	43.9	—	44.54	30.45	—	—	—	32.5	—
15	46.7	48.3	—	47.73	—	—	—	—	—	—

\* The Anthropometric Committee's average includes clothing. In the present investigation the weight of the clothing varied from 1 kgm. in the case of children of five or six years to 3 kgm. for the older ones.

The measurements of the children in height and weight fell so far below the Anthropometric Standard upon which Lusk's coefficients are based, that an independent calculation of the probable basal needs of the various children of each family was made.

The body surface was calculated from the formula

$$S = 0.007184 \times W^{0.425} \times H^{0.725},$$

where  $S$  is the surface in square metres,  $W$  the weight in kilograms, and  $H$  the height in centimetres, and the heat evolved per square metre per hour by the formula  $\log y = 1.8362 - 0.0118x$ , where  $x$  is the age in years and  $y$  the required basal metabolism per hour.

From the total calories consumed during the week by each family were subtracted first the requirements of the parents on the basis that they were persons of average size. The women's food requirements, *i.e.* as eaten, were taken as being equal to 0.83 that of a man at a sedentary occupation, *i.e.* 2500 calories per day. Allowances were made for the men according to their occupations. The basal requirements of the children, calculated as indicated above, were then subtracted, the remainder being the energy available for movement, heat production, muscular work, digestion and growth. The Food (War) Committee of the Royal Society<sup>1</sup> have attempted to deal in this manner with the food requirements of children. Although sufficient data were not forthcoming to allow the Committee to make a definite statement, they tentatively suggest doubling the basal needs for ordinary life and trebling in the case of a very active child.

From the poor physique of the children, in the present investigation, it is apparent either that their food has not been sufficient to allow for normal growth, or that some other factor or factors have inhibited growth. In approaching this question it is necessary to find out whether the diets were the result of unrestricted choice, or whether poverty compelled the mothers to limit the children's food. Each family will be considered by itself.

*A. Families, whose diet has been fairly constant in energy value at the four periods of study.*

									Calories
S 84.	Total calories in 7 days' food	...	...	...	...	...	...	...	95,160
	Father, labourer, at 3500 calories daily	...	...	...	...	...	...	...	24,500
	Mother at 2500 calories daily	...	...	...	...	...	...	...	17,500
	Calories remaining for children	...	...	...	...	...	...	...	53,160
	Basal needs of Alexander (14)	...	...	...	...	...	...	...	8,701
	„ Charlie (11)	...	...	...	...	...	...	...	9,100
	„ Emily (9)	...	...	...	...	...	...	...	7,651
	„ Tommy (7)	...	...	...	...	...	...	...	6,713
	„ David (5)	...	...	...	...	...	...	...	5,999
	„ Baby (3)	...	...	...	...	...	...	...	5,250
	Total basal needs of children for 7 days	...	...	...	...	...	...	...	43,414
	Calories in the food available for energy expenditure in growth, etc.	...	...	...	...	...	...	...	9,746

or 22 per cent. of the basal needs.

All the children are below the Anthropometric Standard Average. Allowing 2 kgs. for clothing their average deficiency in weight is 3.7 kgs. or 13 per cent.

<sup>1</sup> *Report on the Food Requirements of Man and their Variations according to Age, Sex, Size and Occupation, March 1919.*

*Diets of Labouring Class*

	Calories
H 47. Total calories in 7 days' food ... ..	100,894
Father, shoemaker, at 3150 calories daily ... ..	22,050
Mother 2500 calories daily ... ..	17,500
Remainder for children ... ..	61,344
Basal needs of Hannah (13) ... ..	7,903
" Walter (12) ... ..	7,609
" Ina (10) ... ..	6,629
" Jessie (8) ... ..	6,692
" William (6) ... ..	5,600
" Sandy (2) ... ..	5,250
Total basal needs of children for 7 days ... ..	39,683
Calories available in the food for energy expenditure in movement, growth, etc.	21,661

or 55 per cent. of the basal needs.

All the children are below the average in size. Allowing 2 kgms. for clothing, their deficiency in weight averages 6.28 kgms. or 21.5 per cent.

	Calories
M 112. Total calories in 7 days' food ... ..	84,968
Father, seaman, for 1 whole day and 2 meals ... ..	5,830
Mother 2500 calories daily ... ..	17,500
Calories remaining for children ... ..	61,638
Basal needs of James (14) ... ..	8,624
" Sarah (13) ... ..	7,553
" Peggy (13) ... ..	7,490
" Robert (9) ... ..	7,079
" David (7) ... ..	6,895
" Kathie (2) ... ..	5,250
Total basal needs of children for 7 days ... ..	42,891
Calories available in the food for energy expenditure in growth, movement, etc.	18,747

or 44 per cent. of the basal needs.

All the children are undersized. Allowing for clothing as above, they fall short by 8.71 kgms. or 25.5 per cent. of the Anthropometric Committee's averages.

*B. Family, whose diet has varied considerably from time to time.*

	Calories
N 31. Total calories in 7 days' food ... ..	86,769
Mother at 2500 calories daily ... ..	17,500
Calories remaining for children ... ..	69,269
Basal needs of Nettie (15) ... ..	10,881
" Bessie (14) ... ..	10,734
" Alice (13) ... ..	9,043
" Robert (10) ... ..	8,491
" John (9) ... ..	8,958
" Tommy (3½) ... ..	5,600
Total basal needs of children (7 days) ... ..	53,707
Energy in the food remaining for movement, growth, etc. ... ..	15,562

or 29 per cent. of the basal needs.

The two elder girls are above the Anthropometric Committee's average in size, the four younger children are slightly below. The marked fluctuations in energy value of the food consumed by this family were entirely due to changes

in income. When first studied they were in comfortable circumstances. At that time they were consuming, on Atwater's allowances, 4174 calories per man per day. At the second study, owing to the rise which had taken place in the cost of living without a corresponding increase in the family income, they could only afford 3112 calories per man per day. With an improvement in income, when the eldest girl left school, the value of the diet again rose to over 4000 calories, which is equivalent to more than twice the basal needs. In December, while the cost of living had further risen, the eldest girl had had to leave her work to help her mother at home. The Government Separation Allowance was then their only source of income, and this fact at once made its influence felt on the food. In spite of the temporary periods of shortage, the children appear to have suffered little interruption of growth.

Probably in all the foregoing families the metabolism is lower than it would be with a plentiful diet. This would make it possible for them to go on for some time on such small supplies. But if this is so, it will be accompanied by a lessening of the muscular activity necessary to healthy development.

### III. *The cost of living.*

The average value obtained by the five housewives was 210 calories per *ld.* This represents very economical purchasing, the diets including almost no milk, no eggs, little meat, little fish, and little fat. The energy came chiefly from bread and potatoes. Possibly the stunting of growth noticed in the children may be due to a deficiency in food containing the accessory growth-producing factors.

The problem narrows itself down to one of poverty. Only one family (N 150) could afford even the freedom of choice allowed by the rationing scheme. Assuming that children require twice as much food as their basal needs, we can calculate how many calories each mother ought to purchase in food. If divided by 210, the average number of calories purchasable per penny, this will represent the amount which, at the very least, each mother ought to spend on food. More could with advantage be spent so as to allow of more milk, eggs, etc. The following table compares the amount which, from the above calculation, should have been spent on food with the total weekly income of each family:

Table III.

No. of family	Cost of food			Weekly income		
	£	s.	d.	£	s.	d.
S 84	2	11	1	2	15	0
H 47	2	7	4	1	14	0
M 112*	2	3	4	3	1	0
N 31	2	9	11	2	1	6

\* Father intemperate, mother careless.

In addition to food the mother has to make her income cover the costs of rent, coal, gas, clothing, boots, cleansing, and insurance.



## SUMMARY.

1. Throughout the war the food value of the dietaries investigated with one exception showed great constancy, temporary shortage of certain commodities being compensated for by the greater use of others, especially of flour.

2. The food consumed was determined much more by the income and dietary habits of the families than by the restrictions imposed by rationing. The marked variations in the energy value of one dietary from time to time (normally a generous one) were directly caused by changes of income.

3. The children of three families were markedly below the average in height and weight. As the energy available in the food of these families only averaged 40 per cent. above their basal requirements calculated according to age and body surface, it seems probable that the interruption of growth had been caused by an insufficient supply of food.

4. A fourth family had at two periods of study an equally low intake of energy, but during the other two studies had at least 100 per cent. above the basal energy requirements. As the children were normal in development, growth was apparently unchecked by the temporary periods of food shortage.

## A POLYVALENT VACCINE IN THE TREATMENT OF BACILLARY DYSENTERY IN EAST AFRICA<sup>1</sup>.

By W. H. KAUNTZE, M.B.E., M.B., B.Sc. (LOND.),

*Pathologist, East Africa Protectorate  
(late Captain, West African Medical Staff).*

THE research on which this paper is based, was carried out between September 1916, and April 1917, while the writer was in charge of the Carrier Depot Hospital, Nairobi, British East Africa.

Dysentery was the most serious disease met with amongst the porters of the Military Labour Bureau in the early days of the East African Campaign, and was the main cause of death and invaliding even as late as the latter six months of 1916, although stringent sanitary measures had done much to diminish its incidence. Apparently at first it was thought that the disease was amoebic in type, and treatment consisted almost entirely in the administration of emetine. Dr Pirie, Government Pathologist at Nairobi, was the first to point out that this idea was wrong, and in the first eight months of 1916, he conducted a series of bacteriological examinations of the stools of 56 cases of dysentery and showed that in only two cases were amoebae found while dysentery-like bacilli were actually isolated in 35·7 per cent., the main type isolated resembling *B. Shiga*, or frequently *B. Morgan*<sup>2</sup>.

It was just at the conclusion of Dr Pirie's work in June 1916, that the writer took over charge of the Carrier Hospital at Nairobi. From that time the stools of every patient admitted to hospital for dysentery or diarrhoea, were examined by the writer for protozoa, at least twice, and often thrice, as a routine practice, and it was soon apparent from the number of negative results that the conclusions of Dr Pirie were justified, and that we were dealing with an outbreak of dysentery of the bacillary type and not of the amoebic type. This was also borne out by the failure of emetine in the treatment of the disease. As a result of this work the routine administration of emetine ceased, and reliance was placed mainly on saline treatment, though all the usually recommended lines of treatment for bacillary dysentery were tried at one time or other. Still the death rate did not improve as it should have done, and a very large number of cases became more or less chronic. At last it occurred to the writer that a vaccine based on the results of Dr Pirie's research might improve matters, and Dr Ross, the Director of Laboratories,

<sup>1</sup> A preliminary note on this work has appeared in the *British Medical Journal*.

<sup>2</sup> *Journ. of Hygiene*, xv. No. 4.

B.E.A., and Dr Pirie were consulted on the subject. They thought that the idea was worth a trial and agreed to prepare the vaccine. The main difficulty lay in the highly toxic nature of dysentery vaccines prepared in the usual manner, but it was surmounted by Dr Ross's suggestion to sterilise the proposed vaccine by 0·4 per cent. carbolic acid and not by heat. It may be as well to state here, particularly as it is apparently the first occasion on which a dysentery vaccine sterilized in this way has been used on such an extensive scale, that the result was most satisfactory, for in no single instance, either in the prophylactic inoculation of 76,000 porters with the vaccine or in the therapeutic inoculations described in this paper, was an alarming general or even local reaction seen.

The earliest vaccine tried consisted of *B. Shiga* and *B. Flexner* in equal proportions, and as it was uncertain what was the minimum dose that produced effects and what was the maximum dose that could be given with safety, an initial dose of 5 million was tentatively tried. This was cautiously increased as the vaccine showed its value. The original vaccine was brilliantly successful in certain cases and just as distinct a failure in others, although these latter were also of the bacillary type. It was therefore concluded that probably the failures were due to other strains of dysentery bacilli causing the disease in those cases, and consequently the vaccine was made more polyvalent, and finally contained eight strains, namely *B. Shiga* three strains, *B. Flexner* two strains, *B. Morgan* three strains. This was the vaccine used in the cases recorded in the present paper.

Before discussing the results of vaccine therapy in bacillary dysentery, it seems best to give a short account of the disease as seen amongst Africans, in whom it appears to be slightly different in its manifestations and course to the disease amongst Europeans.

#### CAUSES.

In addition to the usually recognized predisposing cause of dysentery, the following factors seem to play an important part in the origination of the disease among Africans.

(1) *Change of Environment.* This factor was particularly noticeable in those recruits who arrived in Nairobi from the region around Lake Victoria Nyanza, involving a train journey of some 250 miles over a water-shed 9000 feet high. In many the disease showed itself during the actual train journey, in others it only appeared after a residence of two or three days in the Carrier Depot at Nairobi. Although in this depot there were recruits from the districts of Kikuyu and Akamba as well as these Lake people the incidence of the disease was distinctly heavier among the latter, and one was forced to conclude that the men were infected before arrival in Nairobi. The foregoing conclusion received support from the fact that this difference in incidence still obtained in Mombasa, to reach which place both Nairobi district and Lake porters had the same rail journey. This therefore raises the question as to whether Lake

tribes are dysentery "carriers," the change in environment leading to an exacerbation of the disease. The writer hopes to be able to throw further light on this suggestion in a later paper.

(2) *Change of Diet.* In the early stages of the campaign it was not sufficiently realized that a native is subject to the same laws of dietetics as an European, and that in his home an immense amount of labour is put into the preparation and thorough cooking of his meal by his wife or wives. When this was understood and a well cooked, well balanced diet provided for the porter, an immediate lessening of intestinal disorders resulted. The African, however, undoubtedly possesses intestines peculiarly liable to be attacked by inflammatory disease caused by errors in diet, resulting in diarrhoea or even dysentery.

(3) *Helminthiasis.* This is extraordinarily prevalent and, in the writer's opinion, is a most important predisposing cause of dysentery (possibly owing to the delicacy of the African's intestines as mentioned above) and in a paper to be published shortly he hopes to bring forward evidence to support this statement. It is sufficient here to give a summary of the helminth infections among the cases on record in this paper as obtained from the results of the examinations of stools.

Table I.

*Helminth Infections in Dysentery Patients.*

Class of case		Total cases examined	Number of species of Helminths observed												Total of infected cases	
			Negative		One		Two		Three		Four		Five			
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Effectives	...	473	42	9	204	43	140	29.5	70	15	15	3	2	0.5	431	91
Invalids	...	272	28	10	100	37	98	36	39	14	7	3	—	—	244	90
Total	...	745	70	9.5	304	40	238	32.75	109	14.5	22	3	2	0.25	675	90.5

In contrast to this, take the following table compiled from the results of autopsies on cases not suffering from dysentery.

Table II.

*Helminth Infections in Non-Dysenteric Patients.*

Class of case			Total cases examined	Number of species of Helminths observed												Total of infected cases	
				Negative		One		Two		Three		Four		Five			
				No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Effectives	}	Invalids	260	80	31	124	48	49	19	7	2	—	—	—	—	180	69
Total			...	260	80	31	124	48	49	19	7	2	—	—	—	—	180

## PATHOLOGY.

One of the most noticeable features about Africans suffering from dysentery is the rapid emaciation which occurs in even moderately bad cases. Forty-eight hours seem sufficient to reduce a patient to a condition of skin and bone. It is a matter of regret that no actual weight statistics are available to illustrate this.

*Central Nervous System.* This usually shows no macroscopical change. In a few cases, however, congestion of the meninges is distinct and the cerebro-spinal fluid is increased in amount. Possibly this may be associated with the acute toxæmia which is always present in these cases.

*Respiratory System.* The lungs, in almost all cases, show signs of congestion and oedema, and it is worthy of note that many cases of dysentery die as the result of a terminal pneumonia. In all the patients in which pneumonia developed in this series, the sputum invariably showed typical pneumococci only. It seems probable that this is a complication of dysentery due to the lowered resistance of the patient (especially as the African seems to be extraordinarily susceptible to pneumococcal infections), and not one of the manifestations of a generalized septicaemia.

*The Circulatory System* shows little change except in a degeneration of the heart muscle which starts as cloudy swelling and in some instances ends in actual fatty degeneration of the myocardium. The heart in most cases is somewhat dilated and often contains an antemortem clot on the right side.

*The Genito-Urinary System* is apparently unaffected, a few cadavers showed congestion of the kidney, which may have had no connection with the original disease.

*The Alimentary System* shows the majority of the lesions in this disease. In the first place the mouth, in very bad cases, may be the site of a certain amount of ulceration, and in an appreciable number of the very toxæmic patients, this developed into cancrum oris, a condition which usually led to the rapid appearance of the man in the mortuary. This lesion seemed in the majority of cases to have developed in the gum in close proximity to the teeth, and spread from there. The pus from these cases showed only the usual pyogenic cocci and some bacilli, both Gram-positive and Gram-negative, but with the means at our disposal they could not be identified.

The stomach, as a rule, was normal in appearance, but in a few isolated cases, in which toxæmia was most distinct, ecchymoses were present in the mucous membrane and in the serous covering.

The small intestines presented a normal appearance, with a few exceptions in which ecchymoses were seen near the lower ends of the ileum, and occasionally slight superficial ulceration was present in the same area. Peyer's patches were not enlarged. The lymphatic glands in the mesentery were usually enlarged and sometimes showed small hæmorrhages in their substance, but no organisms could be isolated from them.

The large intestine was the site of the most extensive and characteristic lesions. As a rule these were confined to the sigmoid and descending colon, but were also found in seven cases in the ascending colon and caecum. No lesion of the appendix was ever observed.

The lesions were divisible into three main types:

Type (1). This was evidently an early form and was seen in cases which succumbed to toxæmia in the very early days of the disease, or in cases which were suffering from some other ailment and in which dysentery had developed in the later stages of that illness. The sigmoid colon and descending colon showed a dark red velvety surface, the mucous membrane being slightly thickened, and here and there small petechial hæmorrhages. The surface of the mucous membrane was covered with a layer of mucus, more or less faecal-stained. The blood vessels running to the affected portion of the large intestine were engorged as a rule and the veins in the actual walls of the intestine were in a condition of venous congestion. No signs of ulceration were present, nor were there any raised yellow nodules on the surface of the intestine suggestive of an amoebic origin of the disease. Sections of the intestine showed engorgement of the mucous cells of the glands of the large intestine, and a diapedesis of polymorpho-nuclear leucocytes into the submucous layer. In three cases a similar appearance was seen in the caecum and ascending colon, either as a concomitant of the disease in the descending colon, or as a solitary lesion.

Type (2). The second stage was evidently only an extension of the first. The lesions were found in the same portions of the intestines, but as a rule occupying a more extended area. The surface of the intestine was covered with a yellowish or dark brown slough, either continuously in the very worst cases, or in patches of varying size. On removal of this slough, an ulcer was left beneath, which, as a rule, presented an appearance of only affecting the mucous membrane and the submucous coat. The edges were irregular in outline, and perpendicular more or less to the floor, which was sometimes rough and sloughy, but usually smooth and formed of the muscular tissue of the intestinal wall. In bad cases the ulceration occasionally extended down to the surface of the serous membrane, but in none of the cases which died, amongst the ones recorded in this paper, was perforation seen. A section of the intestine showed, in the ulcerated area, the usual appearance of coagulation necrosis at the edge of the ulcers, and outside this area the appearance seen in the first stage of the disease described above.

Type (3). The third stage was only seen in chronic cases which had responded to treatment at first, inasmuch as the acute signs of dysentery ceased, but which had developed into a condition of chronic diarrhoea with emaciation. The appearances presented varied enormously. The areas of the intestine affected corresponded to those in the first and second stages. In these cases the large intestine was full of a light-coloured yellowish fluid containing nodules of solid faecal material. Very little mucus was noticeable

on the surface of the intestine. In some cases ulceration of the intestine was still present, but the ulcers were clean with puckered, thickened edges, and apparently in a state where an attempt at healing was taking place. The ulcerated areas were, as a rule, small compared to those in stage 2. The intervening mucous membrane showed scarred areas of fibrous tissue, evidently the site of former ulceration, and much thickening of the remaining unaffected surface. This latter portion showed a velvety appearance with marked rugae. Sections through the ulcers showed regeneration changes in the edges, but, as a rule, little or none in the floor of the ulcer which was practically always formed of the muscular coat.

In other cases no actual ulceration was present, but large areas of the large intestine showed a smooth glazed surface which were the sites of former ulcers, and between these, thickened rugose mucous membrane with markedly velvety appearance. In these cases death was apparently due to loss of fluid owing to a large portion of the large intestine having been thrown out of operation as an absorbing area.

The liver in acute cases was enlarged and engorged. No abscess was seen in it, except in one case which was suffering from *Schistosomum mansoni*, and a scraping of the walls of the abscess showed the eggs of this helminth, but no amoebae.

The spleen in acute cases was enlarged and soft. In chronic cases it was also usually enlarged, but whether this was due to dysentery or to chronic malaria, it would be difficult to say.

The other organs in the alimentary system were normal.

#### SYMPTOMS.

In an African native the symptoms usually associated with dysentery are not so distinct as in the case of Europeans, either from the native dislike of hospital treatment, or from the undoubted fact that, taken as a whole, he is less sensible to pain. The writer personally has seen cases which have gone out to duty involving hard manual labour while suffering with severe dysentery of several days standing, and which, on being called out as suspected cases, have indignantly denied being ill. The consequence of this is that, in a large proportion of cases, men have not come for treatment till the disease is well advanced, and a toxæmic condition already manifest. Dysentery is not usually a disease simulated by the malingerer, owing to the native's dislike of light diet. As a rule the patient only complains of passing blood in the stools, and diarrhoea. On further questioning he may confess to having a certain amount of pain in the stomach and straining at stool. Headache and fever are also complained of in a small proportion of cases.

## PHYSICAL SIGNS.

As has been noted above, the patient often shows signs of the onset of toxæmia in wasting, lethargy, and general weakness. The rapidity with which a native wastes as the result of disease, particularly abdominal disease, is remarkable, and is only equalled by the rapidity with which he puts on flesh once a cure is effected and good diet instituted.

The temperature varies considerably. Some cases never show any rise of temperature from the beginning to the close of the illness, others show an intermittent temperature running up to 101° F. throughout the whole course of the disease, the majority show an initial rise of temperature from 100° F. to 102° F., which, with remissions, lasts for two to three days. A subnormal temperature is usually noticeable before death.

The pulse and respiration rates closely follow the temperature, but as a rule are slightly more rapid than normal during apyrexia, rising to about 80 and 30 respectively.

The tongue is covered with a brownish white fur, but in cases showing distinct toxæmia, it becomes shrunken, covered with brown fur, and is moved with difficulty. The teeth and lips in this toxæmic stage are covered with sordes.

The abdomen as a rule presents no abnormal appearance, though if the disease has been in existence some days, it may be sunken. On palpation, a tender area in the left iliac fossa is discernible. The area of tenderness varies in extent with the severity of the disease, but is usually most marked in the region of the sigmoid colon, and diminishes as one palpates along the length of the descending colon to the splenic flexure. In very bad cases, the colon may be tender and thickened along its whole length.

The stool varies greatly in appearance. The amount of blood may be almost infinitesimal, and in some cases requires a microscope for its detection, or it may constitute the major portion of the evacuation. Mucus is always found if searched for, and usually in fairly large quantities. It is not mixed intimately with the blood, the latter forming streaks on its surface. The actual faecal content of the stool in the early stages of the disease is small, but increases with the administration of purgatives. It is rarely normal in colour, being either paler than usual and even in some cases quite white, or else green. In either case the smell indicates an increase in intestinal fermentation gases. In the very early stages of the disease the stool may be quite formed, the mucus and blood forming a coating for more or less scybalous masses, but this rapidly disappears, and diarrhoea becomes a constant feature. It is very noticeable that the number of stools rarely exceeds 20 a day and is usually not more than 10 to 15.

The liver nearly always shows an increase in size, to about a finger's breadth below the costal margin, and may be slightly tender to the touch. The spleen is rarely affected.



The respiratory system shows no physical sign, unless death is supervening, when indications of hypostatic congestion are found, and in certain cases a terminal lobar pneumonia may end the scene.

The genito-urinary system is not as a rule affected. The urine is usually highly acid, may be small in quantity and consequently highly coloured. Although it was never actually determined, the writer is inclined to believe that the colouring matter is actually increased and not relatively so as the result of concentration.

The blood shows a slight decrease in the content of red blood corpuscles, and a slight polymorphonuclear leucocytosis.

In cases in which toxæmia is marked, the face becomes sunken, the lips and teeth covered with sordes, the tongue covered with brown fur, the abdomen boat-shaped, the patient restless and semi-conscious or delirious. Hiccough then starts, and is nearly always a sign that death is near. In only a few cases when hiccough once sets in, has recovery occurred. In certain patients, hiccough was the first symptom of the onset of toxæmia.

The circulatory system does not, as a rule, show any change. In a few cases when the disease had lasted some considerable time, or toxæmia was well marked, hæmic murmurs appeared, but very little dilatation of the heart was observable antemortem.

#### COMPLICATIONS.

(1) *Cancrum Oris*. This occurred in a few cases in which the dysentery had lasted for some time, and the patient was in a state of chronic toxæmia.

(2) *Perforation*. No case of perforation was ever seen.

(3) *Peritonitis*. No case with peritonitis occurred among these cases.

(4) *Toxæmia*. This is a very frequent complication of bacillary dysentery. Owing to the pressure of routine work, it was impossible to estimate the acidity of the blood in these cases, but it seems probable that an acidosis was responsible for these symptoms. It is hoped in a later paper to elucidate this point. At least it can be recorded here that alkaline treatment gave promise of better results than any other in combating this complication.

(5) *Tissue Desiccation*. This is a frequent cause of death in bacillary dysentery, the constant loss of fluid by the bowel causing a corresponding loss of water by the body cells. Although everything was done to increase the quantity of fluid taken by the patient, it was difficult to overcome the African's rooted idea that the drinking of water leads to increased diarrhoea. Objections were even raised by the natives to fluid diet on this score. Recourse to subcutaneous saline infusion was consequently often necessary.

(6) *Post-Dysenteric Diarrhoea*. Frequently after all signs of dysentery have ceased, an intractable diarrhoea persists, often accompanied by marked toxæmia and wasting. The most probable explanation seems to be that a secondary infection of the ulcers in the large intestine with *B. coli communis* or other intestinal organisms takes place.

## TREATMENT.

Practically every recommended method of treatment was tried during the time the writer was in charge of the hospital. Before his advent, emetine was given to all cases with any symptoms of dysentery, and as far as could be seen from clinical records, without success of any sort. The routine dose was one grain hypodermically once daily for seven to ten doses. Inasmuch as the clinical benefits were negative, and no evidence of amoebic infection was found microscopically or postmortem, the administration of emetine was stopped, except in cases in which amoebae were found by microscopic examination.

Small doses of calomel namely half a grain every hour for eight hours daily for three days, was a form of drug treatment largely used in the earlier cases of this series. Theoretically the idea of stimulating the secretion of bile seems to be an excellent one, inasmuch, as has been mentioned above, the biliary function of the liver seems disorganized in bacillary dysentery, as shown by the green or light coloured stools so frequently seen. Practically it was found that this treatment was too drastic for the majority of the patients suffering from this disease and eventually it was abandoned.

The sulphates of magnesium and sodium were next tried, and it was found that better results were secured by the former salt. Although it is said to exercise a specific effect in bacillary dysentery, this was found not to be the case, as alone without vaccine treatment, it had hardly any perceptible effect on the course of the disease. Combined with vaccine treatment, it was however excellent, and consequently it remained the main drug in the oral treatment of the disease. It may be remarked here that it seems strange that sodium sulphate, which is believed to act more strongly on the biliary secretion than magnesium sulphate, should have a weaker therapeutic action than the latter, but such is the practical experience in this series of cases. Other drugs tried for oral treatment were *mistura chlorinata* consisting of potassium chlorate  $2\frac{1}{2}$  grains, acid hydrochloric pure  $3\frac{1}{2}$  minims, quinine hydrochloride 3 grains, syrup of lemon 40 minims, water to 1 ounce, given every four hours, this being without any appreciable effect on the disease, and castor oil in 4 drachm doses thrice daily also with results distinctly inferior to those of magnesium sulphate.

In addition to these medicines, the object of which was either to wash out the toxins from the bowel or to inhibit bacterial development in the intestines, other drugs were used. Thus, as a result of the discovery of the vast number of dysentery patients suffering from worms (one patient passed a *Taenia saginata* which was rolled into a ball the size of a football, took 20 minutes to pass, and when laid out was 250 feet long and only one head was found in the whole mass; another passed 80 *Ascaris lumbricoides*, 30 on the first day and 50 on the second), anti-helminthics were always administered directly a patient entered hospital, and repeated at intervals till microscopic examination showed that the stools were negative. Of the anti-helminthics, thymol was found to

be the best, given in three doses of 30 grains at intervals of 2 hours on an empty stomach, followed by a dose of magnesium sulphate. Of the others, beta-naphthol was found the second best, while santonin proved the most effective in the treatment of ascariasis. Thymol was equally effective with tapeworms as with ankylostomes, surpassing Felix mas in the elimination of the former infection.

At a late stage of this series, the writer's attention was drawn to a note in the *British Medical Journal* relating to the use of Tinctura Rhei Composita in post-dysenteric diarrhoea. This drug was tried in three cases, and found so efficient in 4 drachm doses repeated at intervals of four days when necessary, that it became a routine treatment, and in at least two cases of children suffering from dysentery, it was found to effect a cure quite apart from other treatment.

As mechanical methods for cleaning the inflamed bowel and diminishing toxin absorption, rectal irrigations of sodium carbonate, potassium permanganate, tannic acid, silver nitrate, and protargol were all tried in succession, but it must be confessed without any improvement in the case. It seems probable that the injection, inasmuch as it could only be given by native orderlies, failed to reach the inflamed portion of the bowel, and was so disliked by the African that the mental depression it produced probably more than counteracted any good done by the actual irrigation.

As regards complications tissue desiccation was met by enforcing the taking of fluid by mouth, and when this was not enough, by subcutaneous saline infusions. In the later cases of the series, when the condition of the patients was extremely critical even when admitted, the latter treatment was called for from the first, and in many cases undoubtedly saved the patient's life. In the light of later knowledge, however, the writer would use an isotonic solution of sodium bicarbonate, and would be tempted to try Professor Bayliss's 6 per cent. gum arabic in this solution. The infusion acts partly by replacing lost tissue fluid, partly by raising the blood pressure and partly by diluting the toxins in the blood.

Toxaemia was combated by the administration of potassium bicarbonate in 30 gr. doses four times daily, and when the potassium salt was not available, that of sodium. This was based on a supposition yet unproved that the toxæmia was due to acidosis. Certainly in some cases it did good, and was one of the most effective drugs in relieving hiccough, which again was probably only a result of acidosis. As a further attempt to diminish the absorption of toxins from the bowel, caecostomy was performed in two cases and continuous saline irrigation instituted. Unfortunately though both cases survived the actual operation, it was apparently performed at too late a stage of the disease, and both succumbed to toxæmia. The main difficulty in this treatment seems to be to select cases late enough to justify the performance of the operation, yet early enough to give the patient a reasonable hope of recovery. Still it seems better to operate too early than too late.

For hiccough, potassium bromide in 10 gr. doses thrice daily, and a mustard plaster on the epigastrium seemed to be the most effective measures of treatment. Tinctura iodi in one minim doses in water every hour for six hours was very useful in certain obstinate cases.

Cancrum Oris was always fatal until it was treated by a potassium chlorate gargle combined with the internal administration of 15 gr. of potassium chlorate thrice daily. Under this treatment about 50 per cent. of the cases recovered.

Post-dysenteric diarrhoea was treated with considerable success by giving a vaccine of *B. coli communis* (2000 million per c.c.) in doses of  $\frac{1}{2}$  c.c., 1 c.c., and 2 c.c. at intervals of three days, Tinctura Rhei Composita in 4 drachm doses at intervals of four days was also useful in certain cases.

### DIET.

Copies of two of the latest hospital dietaries are attached. In these dietaries Diet B combines the Fluid Diet and Semi-solid Diet of the dietary in force when the cases referred to in this paper were in hospital.

#### DIET I.

DIET A			DIET B		Cooking instructions
(Green Ticket)		Hour	(Yellow Ticket)		
Milk	8 oz.	2 a.m.	Milk	10 oz.	<i>Milk.</i> All milk must be boiled and served to the patients <i>warm</i> .
Brandy	$\frac{1}{2}$ "	4 a.m.	Sugar	1 "	
Sugar	$\frac{1}{2}$ "	5 a.m.	Milk	10 oz.	
			Sugar	1 "	<i>Arrowroot.</i> This must be boiled with milk and sugar for half an hour.
Tea	1 pint	6 a.m.	Tea	1 pint	
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 "		Milk	2 "	<i>Matama Uji.</i> This must be boiled for two hours, forming a thin gruel.
Arrowroot	4 oz.	8 a.m.	Matama uji	6 oz.	
Milk	2 "				
Sugar	$\frac{1}{2}$ "				<i>Rice Jelly.</i> The rice must be cooked for at least three hours, or longer if the rice has not reached the stage of jellification.
Milk	4 oz.	10 a.m.	Milk	4 oz.	
Brandy	$\frac{1}{2}$ "		Sugar	$\frac{1}{2}$ "	
Soup	1 pint	12 noon	Rice jelly	8 oz.	
Bread	4 oz.		Milk	4 "	
Milk	4 oz.	2 p.m.			
Brandy	$\frac{1}{2}$ "				
Tea	1 pint	4 p.m.	Tea	1 pint	
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 "		Milk	2 "	
Arrowroot	4 oz.	5 p.m.	Matama uji	6 oz.	
Milk	2 "				
Sugar	$\frac{1}{2}$ "				
		6 p.m.			
Milk	8 oz.	8 p.m.			
Brandy	$\frac{1}{2}$ "	10 p.m.	Milk	10 oz.	
Sugar	$\frac{1}{2}$ "		Sugar	1 "	
		12 midnight			

Uji is a thin gruel.

Ugali is a very thick porridge.

*Bacillary Dysentery*DIET I (*cont.*).

## DIET C. Full (White Ticket).

Monday, Wednesday, Friday, and Sunday.

Diet per man		Cooking pots per 500 men	Hour	Cooking instructions
		3 of 25 gals.	2 a.m.	Boil water.
			3 a.m.	Put in crushed mealies 12 oz. per man and 1 lb. salt per 25 gallon pot. Boil fiercely till 10 a.m.
		2 of 25 gals.	4 a.m.	Put 4 oz. beans per man and $\frac{3}{4}$ lb. salt per 25 gallon pot into cold water, bring to boil and boil hard till 11 a.m.
Tea	1 pint	1 of 100 gals.	5.30 a.m.	Boil water, make tea 1 pint per man with 2 oz. sugar per man. Serve at 6 a.m.
Sugar	2 oz.	2 of 25 gals. (matama)	6 a.m.	Put 6 oz. matama flour per man into some boiling water without salt and boil till 8 a.m. making uji.
		5 of 5 gals. (rice)		Put 2 oz. per man of rice into cold water with 1 oz. salt per 5 gallon dixie and boil till 7.30 a.m., then evaporate off water over slow fire.
Matama	6 oz.		8 a.m.	Serve matama uji with rice.
Rice	2 "	1 of 25 gals. (meat)	9 a.m.	Put meat $\frac{1}{2}$ lb. per man with 1 lb. salt per 25 gallon pot into some boiling water.
		1 of 25 gals. (bones)		Put bones from this meat into cold water and boil up.
				Boil meat and bones till 12 noon, mix soup from each, and give 1 pint per man.
			10 a.m.	Pour off water from crushed mealies, transfer latter to small dioxies, evaporate off remaining water over slow fire.
		2 of 25 gals.	10.30 a.m.	Put peeled bananas $\frac{1}{2}$ lb. per man with $\frac{1}{2}$ lb. salt per 25 gallon pot into boiling water, boil till 11.30 a.m., pour off water and beat bananas into a mash.
Bananas	$\frac{1}{2}$ lb.	2 of 25 gals.	11.30 a.m.	Pour off water from beans, put latter in small dioxies over a slow fire to evaporate off rest of water.
Mealies	$\frac{1}{4}$ "			
Beans	$\frac{1}{4}$ "			
Meat	$\frac{1}{2}$ "		12 noon	Mix beans, crushed mealies and banana mash into a thick paste and serve with meat and soup.
Soup	1 pint			
			3 p.m.	Put washed unpeeled sweet potatoes 3 lb. per man without salt into cold water, boil till 4.30 p.m., then pour off water and steam over a slow fire.
Sweet potatoes	3 lb.		5 p.m.	Serve sweet potatoes.

*Alternatives.*

1. Matama flour, mwele flour, wimbe flour and mohogo meal are all interchangeable when made into uji.

2. Crushed mealies can be replaced by mealie meal ugali. For this mealie meal need only be boiled for five hours but should be treated exactly the same as crushed mealies otherwise.

3. Sweet potatoes may be replaced by ordinary potatoes 2 lb.

DIET I (*cont.*).

DIET C. Full (White Ticket).

Tuesday, Thursday and Saturday.

Diet per man		Cooking pots per 500 men	Hour	Cooking instructions
Tea	1 pint }	1 of 100 gals.	5.30 a.m.	Boil water, make tea 1 pint per man with 2 oz. sugar per man. Serve at 6 a.m.
Sugar	2 oz. }	2 of 25 gals. (matama)		Put 6 oz. matama flour per man in some boiling water without salt and boil till 8 a.m. making uji.
		5 of 5 gals. (rice)		Put 2 oz. of rice per man in cold water with 1 oz. of salt per 5 gallon dixie and boil till 7.30 a.m., then evaporate off water over slow fire.
Matama uji	6 oz. }	2 of 25 gals.	8 a.m.	Serve matama uji with the rice.
Rice	2 „ }		„	Put crushed mealies 6 oz. per man in boiling water with 1 lb. salt per 25 gallon pot. Boil hard till 3 p.m.
		1 of 25 gals.	8.30 a.m.	Put 2 oz. beans per man and $\frac{3}{4}$ lb. salt per 25 gallon pot into cold water, bring to boil and boil hard till 4 p.m.
		1 of 25 gals. (meat)	9 a.m.	Put meat $\frac{1}{2}$ lb. per man with 1 lb. salt per 25 gallon pot into boiling water.
		1 of 25 gals. (bones)		Put bones from this meat into cold water and boil up.
				Boil meat and bones till 12 noon, mix soup from each and give 1 pint per man.
		22 of 5 gals. (dixies)	10 a.m.	Put rice 12 oz. per man into boiling water in dixies. Boil and stir well till 11.30 a.m. Add 1 oz. salt per dixie and $\frac{1}{2}$ oz. ghee per man at 10.30 a.m.
			11.30 a.m.	Put rice over small fire to evaporate off water.
Rice	12 oz. }			
Ghee	$\frac{1}{2}$ „ }		12 noon	Serve rice, meat and soup.
Meat	$\frac{1}{2}$ lb. }			
Soup	1 pint }		3 p.m.	Pour off water from crushed mealies, transfer latter to small dixies and evaporate off remaining water over slow fire.
		1 of 25 gals.	3.30 p.m.	Put peeled bananas $\frac{1}{4}$ lb. per man with $\frac{1}{2}$ lb. salt per 25 gallon pot into boiling water, boil till 4.30 p.m., pour off water and beat into mash.
			4 p.m.	Pour off water from beans, put latter into dixies over a slow fire to evaporate off remaining water.
			4.30 p.m.	Mix beans, crushed mealies and banana mash into a thick paste.
Bananas	$\frac{1}{4}$ lb. }		5 p.m.	Serve the mixed beans and mealies.
Mealies	6 oz. }			
Beans	2 oz. }			

*Alternatives.*

1. Matama flour, mwele flour, wimbe flour and mohogo meal are all interchangeable when made into uji.

2. Crushed mealies can be replaced by mealie meal ugali. For this mealie meal need only be boiled for five hours but should be treated exactly the same as crushed mealies otherwise.

*Bacillary Dysentery*

## DIET II.

DIET A			DIET B		
(Green Ticket)		Hour	(Yellow Ticket)	Cooking instructions	
Milk	8 oz.	2 a.m.	Milk	10 oz.	All milk must be boiled and served to the patient <i>warm</i> .
Brandy	$\frac{1}{2}$ "		Sugar	1 "	
Sugar	$\frac{1}{2}$ "				
Milk	8 oz.	4 a.m.			<i>Arrowroot</i> . This must be boiled with milk and sugar for half an hour.
Brandy	$\frac{1}{2}$ "				
Sugar	$\frac{1}{2}$ "				
Milk	8 oz.	5 a.m.	Milk	10 oz.	4 ounces making 2 pints.
Brandy	$\frac{1}{2}$ "		Sugar	1 "	
Sugar	$\frac{1}{2}$ "				
Tea	1 pint	6 a.m.	Tea	1 pint	<i>Mealie meal uji</i> . This must be boiled for two hours forming a thin gruel.
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 "		Milk	2 "	
Arrowroot	4 oz.	8 a.m.	Mealie meal uji	6 oz.	<i>Rice Jelly</i> . The rice must be cooked for at least three hours or longer if the rice has not reached the stage of jellification.
Milk	2 "		Sugar	1 "	
Sugar	$\frac{1}{2}$ "				
Milk	4 oz.	10 a.m.	Milk	4 oz.	
Brandy	$\frac{1}{2}$ "		Sugar	$\frac{1}{2}$ "	
Soup	1 pint	12 noon	Milk	4 oz.	
Bread	4 oz.		Rice jelly	8 "	
Milk	4 oz.	2 p.m.			
Brandy	$\frac{1}{2}$ "				
Tea	1 pint	4 p.m.	Tea	1 pint	
Sugar	1 oz.		Sugar	1 oz.	
Milk	4 "		Milk	2 "	
Arrowroot	4 oz.	5 p.m.	Mealie meal uji	6 oz.	
Milk	2 "		Sugar	1 "	
Milk	8 oz.	6 p.m.			
Brandy	$\frac{1}{2}$ "				
Sugar	$\frac{1}{2}$ "				
Milk	8 oz.	8 p.m.			
Brandy	$\frac{1}{2}$ "				
Sugar	$\frac{1}{2}$ "				
Milk	8 oz.	10 p.m.	Milk	10 oz.	
Brandy	$\frac{1}{2}$ "		Sugar	1 "	
Sugar	$\frac{1}{2}$ "				
Milk	8 oz.	12 midnight			
Brandy	$\frac{1}{2}$ "				
Sugar	$\frac{1}{2}$ "				

Uji is a thin gruel.

Ugali is a very thick porridge.

## DIET C. (White Ticket.)

Diet per man	Cooking pots per 500 men	Hour	Cooking instructions
Tea 1 pint	1 of 100 gals.	5.30 a.m.	Boil water, make tea 1 pint per man with 2 oz. sugar per man. Serve at 6 a.m. with 1 oz. milk.
Sugar 2 oz.			
Milk 1 "			
	2 of 25 gals. (mealie meal uji)	6 a.m.	Put 6 oz. mealie meal flour per man in boiling water and $\frac{1}{2}$ lb. salt per 25 gallon pot and boil till 8 a.m. forming a thin gruel.
	5 dixies rice (5 gals.)	6 a.m.	Put 2 oz. rice per man in cold water with 1 oz. salt per 5 gallon dixie and boil till 7.30 a.m. Then evaporate off water over a slow fire.

DIET II (*cont.*).

Diet per man		Cooking pots per 500 men	Hour	Cooking instructions
Mealie uji	6 oz. }		8 a.m.	Serve mealie meal uji with rice.
Rice	2 „ }	2 of 25 gals. 1 of 25 gals.	8.30 a.m.	Put 6 oz. beans per man into cold water and boil hard till 11.30 a.m. Pour off water and serve at 12 noon.
			10 a.m.	Put rice 12 oz. per man into boiling water in dixies, boil and stir well till 11.30 a.m. Add salt 1 oz. per dixie and $\frac{1}{2}$ oz. ghee per man at 10.30 a.m.
			11.30 a.m.	Put rice over a small fire to evaporate off water.
Rice	12 oz. }		12 noon	Serve rice and beans.
Ghee	$\frac{1}{2}$ „ }			
Beans	6 „ }	2 of 25 gals. 1 of 25 gals. (meat) 1 of 25 gals. (bones)	2 p.m.	Put meat $\frac{1}{2}$ lb. per man with 1 lb. salt per 25 gallon pot into boiling water. Put bones from this meat into cold water and boil. Boil meat and bones till 5 p.m. Mix soup from each and give 1 pint per man.
			3 p.m.	Put 6 oz. mealie meal flour per man in boiling water and $\frac{1}{2}$ lb. per 25 gallon pot and boil till 5 p.m. forming a thin gruel.
Mealie meal uji	6 oz. }		5 p.m.	Serve mealie meal uji and meat and soup.
Meat	$\frac{1}{2}$ lb. }			
Soup	1 pint }			

The patient was treated with a fluid diet until his stools had been solid for two days and without any suspicion of blood or mucus. He was then put on to the semi-solid diet for two days, and if no diarrhoea showed itself in this time, he went on to full diet.

## GENERAL TREATMENT.

All dysentery cases were kept in bed as far as possible during the acute stage of the disease. Their mouths were cleaned twice daily with potassium permanganate lotion, and, if necessary, also in the intervals between these routine washings. They were also given a pint of water six times daily in addition to the fluid matter of the diet.

Most of these methods of treatment of bacillary dysentery by means of drugs were tried before vaccine treatment was commenced, and although a certain amount of improvement was attained in the reduction of the death and invaliding rate, yet as will be shown later from the results of treatment in 138 cases without anti-dysenteric vaccine, the results were far from encouraging.

## TREATMENT WITH ANTI-DYSENTERIC VACCINE.

The vaccine as used in this series of cases consisted of:

- (1) *B. Shiga* (3 strains) 500 millions.
- (2) *B. Flexner* (2 strains) 250 millions.
- (3) *B. Morgan* (3 strains) 750 millions.

Each strain was inoculated separately into a flask containing peptone bouillon, incubated for 72 hours, and then killed by the addition of 0.5 per



cent. carbolic acid. Each flask was then allowed to stand for 48 hours, and at the end of this time was tested for sterility. If sterile, the dead cultures were standardized, mixed in the above proportion, and bottled.

In order to compare the results of vaccine treatment, it was found necessary to record the cases under different groups, each group of cases being treated with a different dose of vaccine. As some of these groups contained only a very small number of cases, too inadequate to form any conclusions on, the groups were re-collected into two main divisions, namely *Division X* (Tables III–VI) into which all the early cases fell, and in which the dosage in the light of later experience was small, and *Division Y* (Tables VII–XIII), into which all the later cases fell, and in which the dosage was much larger.

It was further found that cases had to be divided into two main classes, namely “Effectives,” and “Invalids,” to admit of adequate comparison, “Effectives” being porters who were actually doing duty at the time of the onset of dysentery, or who had been passed as recruits for work: “Invalids” being porters who had been returned from posts nearer the actual fighting line, for disease contracted in the field which rendered them unfit for further duty. Thus under each group will be found two main classes, namely Effectives and Invalids.

Again it was obvious that it was unfair to take each case of dysentery as the same in severity, for some men reported sick as soon as the disease showed itself, others not for ten days afterwards, when toxaemia had set in and the patient had been reduced to a very weak state. The disease itself was also not always equal in severity. Consequently it was decided that some sort of subdivision of cases according to the severity of the disease and to the condition of the patient was essential.

The severity of the attack was adjudged on four main points, namely the symptoms, the amount of toxaemia, the amount of blood and mucus in the stools, and the number of evacuations daily.

The following classification was adopted:

(a) *Slight cases* in which the patient's physical condition was good, and the disease was mild and unaccompanied by toxaemia.

(b) *Medium cases* in which the patient's physical condition was good, but the disease was more severe, and in which a certain degree of toxaemia was seen.

(c) *Bad cases* in which the patient's physical condition was fair, but in which the disease was severe, and toxaemia marked.

(d) *Very bad cases* in which the patient's physical condition was poor, and in which the disease was severe and marked toxaemia was present.

(e) *Hopeless cases* in which the patient was admitted either in a comatose condition, or in which the patient was so emaciated and toxaemia so marked that no hope of the patient's recovery could be entertained.

It must here be remarked that the standard of physical condition was of necessity made much lower for invalids than for effectives, and that even then

possibly a considerably larger proportion of them should have been included amongst the "hopeless" class, but in every case the most optimistic view possible was taken.

A classification based on the characters of the case and the condition of the patient necessarily depends largely on the judgment of the observer, consequently in each group, a total is made of all cases included in that group, so that conclusions based on these totals are absolutely independent of any personal bias. It is unfortunate that whereas the vaccine cases were consecutive admissions to hospital, no case being excluded, the only non-vaccine cases available for comparison of which a record exists, were more or less selected men, in that all were "Effectives" and "Hopeless" cases were definitely excluded, this series having been recorded before vaccine treatment was started or even thought of. Consequently in order to compare total cases, it has been found essential to show in Table XV not only totals showing all cases, but also totals including all cases but "hopeless" ones.

As a further note it may be stated that even a comatose "hopeless" case sometimes recovered consciousness, and with the aid of stimulants and intravenous salines lived sometimes for a considerable number of days after admission, but in not a single case was the classification of "hopeless," which was always made on the day of admission, found unjustified, for no case in this class ever survived to be cited as a recovery.

A further division of porters was also necessary for statistical purposes. A certain number of porters received a dose of 1 c.c. (1500 million) of anti-dysenteric vaccine, others received a dose of 4 c.c. of anti-dysenteric vaccine, in both cases as a prophylactic measure when recruited. A large proportion of the cases received no prophylactic inoculation at all.

We have therefore the following outline classification under which all cases are brought.

- (i) *Division.* X or Y, *i.e.* small or large therapeutic doses vaccine administered.
- (ii) *Group.* I—X depending on therapeutic dose of vaccine administered.
- (iii) *Class.* I. Effectives, II. Invalids.
- (iv) *Sub-Class.*
  - A. Receiving no prophylactic inoculation.
  - B. Receiving 1 c.c. A.D. vaccine as a prophylactic.
  - C. Receiving 4 c.c. A.D. vaccine as a prophylactic.
- (v) *Character of Case.* As described above.



Table V.  
 GROUP B II. Initial dose. 90 million. (Doses every second day increased by half as much again.)

Class	Character of case	Recoveries										Average number of days in hospital					
		Total		To duty		Invalided		Deaths		Duty		Invalided		Died		All cases	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>Effectives</i> Prophylactic inoculation Nil	Slight	5	100-00	4	80-00	1	20-00	—	—	—	—	8-00	9-00	—	—	8-20	—
	Medium	3	100-00	2	66-67	1	33-33	—	—	—	—	8-50	13-00	—	—	10-00	—
	Bad	10	90-00	6	60-00	3	30-00	1	10-00	19-50	26-33	19-00	21-50	—	—	21-50	—
	Very bad	8	62-50	3	37-50	2	25-00	3	37-50	19-67	39-50	27-00	27-37	—	—	27-37	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total ...	26	84-61	15	57-69	7	26-92	4	15-39	15-00	25-71	25-00	19-42	—	—	19-42	—
<i>Invalids</i> Prophylactic inoculation Nil	Slight	9	88-89	2	22-22	6	66-67	1	11-11	12-50	14-50	1-00	12-55	—	—	12-55	—
	Medium	2	50-00	1	50-00	—	—	1	50-00	10-00	—	18-00	14-00	—	—	14-00	—
	Bad	8	40-00	2	25-00	2	25-00	4	50-00	34-00	8-50	24-75	23-00	—	—	23-00	—
	Very bad	10	40-00	—	—	4	40-00	6	60-00	—	78-25	19-83	43-20	—	—	43-20	—
	Hopeless	3	—	—	—	—	—	3	100-00	—	—	24-00	24-00	—	—	24-00	—
	Total ...	32	53-12	5	15-62	12	37-50	15	46-88	20-60	34-75	20-60	25-91	—	—	25-91	—

Table VI.  
 GROUP B III. Initial dose. 90 million. (Doses repeated weekly increased by half as much again.)

Class	Character of case	Recoveries										Average number of days in hospital				
		Total cases		Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases	
		No.	%	No.	%	No.	%	No.	%	No.	%					
<i>Effectives</i> Prophylactic inoculation Nil	Slight	2	—	2	100-00	2	100-00	—	—	—	—	9-00	—	—	—	9-00
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	2	—	—	—	—	—	—	—	2	100-00	—	—	—	52-50	52-50
	Hopeless	1	—	—	—	—	—	—	—	1	100-00	—	—	—	29-00	29-00
	Total ...	5	—	2	40-00	2	40-00	—	—	3	60-00	9-00	—	—	44-66	30-40
<i>Invalids</i> Prophylactic inoculation Nil	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	1	—	1	100-00	—	—	1	100-00	—	—	—	27-00	—	—	27-00
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total ...	1	—	1	100-00	—	—	—	—	1	100-00	—	—	27-00	—	27-00

Table VII. Group B iv. Initial dose. 180 million. (Doses repeated weekly increased by half as much again.)

Class	Character of case	Recoveries						Deaths				Average number of days in hospital		
		Total		To duty		Invalided		No.		%		Duty	Invalided	Died
		No.	%	No.	%	No.	%	No.	%	No.	%			
<i>Effectives</i>	Slight	5	100-00	4	80-00	1	20-00	—	—	—	—	16-00	10-00	—
	Medium	5	100-00	4	80-00	1	20-00	—	—	—	—	9-25	11-00	—
	Bad	4	100-00	3	75-00	1	25-00	—	—	—	—	14-67	35-00	—
	Very bad	4	100-00	2	50-00	—	—	—	—	—	—	49-00	—	25-50
<i>Nil</i>	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	37-25
Total ...		18	88-89	13	72-22	3	16-67	2	11-11	—	—	18-69	18-67	25-50
<i>Invalids</i>	Slight	1	100-00	1	100-00	—	—	—	—	—	—	8-00	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	2	100-00	—	—	2	100-00	—	—	—	—	—	29-00	—
<i>Nil</i>	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	29-00
Total ...		3	100-00	1	33-33	2	66-67	—	—	—	—	8-00	29-00	—

Table VIII. Group B v. Initial dose. 750 million. (Doses doubled at weekly intervals.)

Class	Character of case	Recoveries						Deaths				Average number of days in hospital		
		Total		To duty		Invalided		No.		%		Duty	Invalided	Died
		No.	%	No.	%	No.	%	No.	%	No.	%			
<i>Effectives</i>	Slight	4	100-00	4	100-00	—	—	—	—	—	—	7-50	—	7-50
	Medium	3	100-00	3	100-00	—	—	—	—	—	—	12-33	—	12-33
	Bad	4	100-00	4	100-00	—	—	—	—	—	—	11-25	—	11-25
	Very bad	2	100-00	1	50-00	—	—	1	50-00	—	—	52-00	—	33-00
<i>Nil</i>	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	42-50
Total ...		13	92-30	12	92-30	—	—	1	7-70	—	—	13-66	—	33-00
<i>Effectives</i>	Slight	1	100-00	1	100-00	—	—	—	—	—	—	13-00	—	13-00
	Medium	1	100-00	—	—	—	—	1	100-00	—	—	—	—	18-00
	Bad	2	100-00	2	100-00	—	—	—	—	—	—	14-50	—	14-50
	Very bad	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Nil</i>	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		4	75-00	3	75-00	—	—	1	25-00	—	—	14-00	—	18-00
<i>Invalids</i>	Slight	3	100-00	—	—	1	33-33	2	66-67	—	—	—	9-00	9-33
	Medium	1	100-00	1	100-00	—	—	—	—	—	—	33-00	—	33-00
	Bad	7	85-71	5	71-43	1	14-28	1	14-28	—	—	43-20	65-00	41-57
	Very bad	1	100-00	—	—	—	—	1	100-00	—	—	—	12-00	12-00
<i>Nil</i>	Hopeless	2	100-00	—	—	—	—	2	100-00	—	—	—	—	49-00
Total ...		14	57-14	6	42-86	2	14-28	6	42-86	—	—	41-50	37-00	23-17

Table IX.

GROUP B VI. Initial dose. 1500 million. (Doses doubled at weekly intervals.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases		
		No.	%	No.	%	No.	%	No.	%						
<i>Effectives</i> Prophylactic inoculation Nil	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	7	100-00	7	100-00	—	—	—	—	16-71	—	—	16-71	—	—
	Bad	2	100-00	2	100-00	—	—	—	—	11-00	—	—	11-00	—	—
	Very bad	3	100-00	3	100-00	—	—	—	—	24-67	—	—	24-67	—	—
	Hopeless	3	—	—	—	—	—	3	100-00	—	—	60-33	60-33	—	—
Total ...		15	12	80-00	12	80-00	—	3	20-00	17-75	—	60-33	60-33	—	26-26
<i>Effectives</i> Prophylactic inoculation 1 c.c.	Slight	1	100-00	1	100-00	—	—	—	—	15-00	—	—	15-00	—	—
	Medium	13	100-00	13	100-00	—	—	—	—	12-46	—	—	12-46	—	—
	Bad	6	83-33	5	83-33	—	—	1	16-67	25-60	—	9-00	22-83	—	—
	Very bad	7	71-43	5	71-43	—	—	2	28-57	41-40	—	31-50	38-56	—	—
	Hopeless	3	—	—	—	—	—	3	100-00	—	—	12-00	12-00	—	—
Total ...		30	24	80-00	24	80-00	—	6	20-00	21-33	—	36-00	36-00	—	20-67
<i>Invalids</i> Prophylactic inoculation Nil	Slight	2	100-00	—	—	2	100-00	—	—	—	9-50	—	9-50	—	—
	Medium	14	100-00	9	64-29	5	35-71	—	—	16-44	21-40	—	18-21	—	—
	Bad	11	81-82	6	54-54	3	27-28	2	18-18	38-67	31-33	51-00	38-91	—	—
	Very bad	12	33-33	3	25-00	1	8-33	8	66-67	31-33	13-00	28-75	28-08	—	—
	Hopeless	6	—	—	—	—	—	6	100-00	—	—	21-83	21-83	—	—
Total ...		45	29	64-44	18	40-00	11	24-44	16	35-56	26-33	21-18	28-94	26-00	—

Table X. Group B vii. Initial dose. 3000 million. (Doses doubled at weekly intervals.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total cases		Total		To duty		Invalided		Deaths		Duty	Invalided	Died	All cases
		No.	%	No.	%	No.	%	No.	%	No.	%				
<i>Effectives</i> Prophylactic inoculation Nil	Slight	2	2	100-00	2	100-00	—	—	—	—	—	8-50	—	—	8-50
	Medium	8	8	100-00	8	100-00	—	—	—	—	—	19-00	—	—	19-00
	Bad	12	11	91-67	11	91-67	—	—	—	1	8-33	18-09	—	53-00	21-00
	Very bad	17	13	76-47	12	70-59	1	5-88	4	23-53	25-42	48-00	—	27-50	27-50
	Hopeless	3	—	—	—	—	—	—	—	3	100-00	—	—	16-66	16-66
Total ...		42	34	80-95	33	78-75	1	2-38	8	19-05	20-39	48-00	26-62	22-24	22-24
<i>Effectives</i> Prophylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	2	2	100-00	2	100-00	—	—	—	—	—	18-50	—	—	18-50
	Bad	5	5	100-00	5	100-00	—	—	—	—	—	16-80	—	—	16-80
	Very bad	8	5	62-50	4	50-00	1	12-50	3	37-50	21-00	53-00	50-33	—	36-00
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		15	12	80-00	11	73-33	1	6-67	3	20-00	18-64	53-00	50-33	27-27	27-27
<i>Effectives</i> Prophylactic inoculation 4 c.c.	Slight	3	3	100-00	3	100-00	—	—	—	—	10-67	—	—	—	10-67
	Medium	24	21	87-50	21	87-50	—	—	—	3	12-50	17-67	—	39-00	20-33
	Bad	51	44	86-27	42	82-35	2	3-92	7	13-73	29-62	86-00	—	20-57	24-82
	Very bad	53	37	69-81	35	66-04	2	3-77	16	30-19	29-00	98-00	—	33-19	30-98
	Hopeless	7	—	—	—	—	—	—	7	100-00	—	—	—	17-86	17-86
Total ...		138	105	76-09	101	73-19	4	2-90	33	23-91	23-45	92-00	27-79	25-75	25-75
<i>Invalids</i> Prophylactic inoculation Nil	Slight	2	2	100-00	2	100-00	—	—	—	—	7-50	—	—	—	7-50
	Medium	3	3	100-00	3	100-00	—	—	—	—	13-67	—	—	—	13-67
	Bad	3	2	66-67	2	66-67	—	—	1	33-33	22-50	—	—	8-00	17-67
	Very bad	6	2	33-33	2	33-33	—	—	4	66-67	36-00	—	—	27-25	30-17
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		14	9	64-29	9	64-29	—	—	5	35-71	19-22	—	23-40	20-71	20-71
<i>Invalids</i> Prophylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	4	2	50-00	1	25-00	1	25-00	2	50-00	30-00	75-00	15-50	—	34-00
	Bad	2	1	50-00	1	50-00	—	—	1	50-00	13-00	—	—	9-00	11-00
	Very bad	14	3	21-43	2	14-29	1	7-14	11	78-57	20-50	73-00	10-64	24-36	24-36
	Hopeless	3	—	—	—	—	—	—	3	100-00	—	—	7-33	7-33	7-33
Total ...		23	6	26-09	4	17-39	2	8-70	17	73-91	21-00	74-00	17-00	22-65	22-65
<i>Invalids</i> Prophylactic inoculation 4 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	3	100-00	3	100-00	—	—	—	—	35-33	—	—	—	35-33
	Bad	10	5	50-00	3	30-00	2	20-00	5	50-00	16-87	52-50	24-60	27-80	27-80
	Very bad	11	3	27-27	3	27-27	—	—	8	72-73	49-00	—	22-63	29-82	29-82
	Hopeless	9	—	—	—	—	—	—	9	100-00	—	—	9-00	9-00	9-00
Total ...		33	11	33-33	9	27-27	2	6-06	22	66-67	33-67	52-50	17-50	24-03	24-03

Table XI.  
GROUP B VIII. Initial dose. 6000 million. (Doses doubled at weekly intervals.)

Character of case		Recoveries										Average number of days in hospital					
		Total			To duty			Invalided			Deaths						
		No.	%		No.	%		No.	%		No.	%		Duty	Invalided	Died	All cases
<i>Effectives</i> Propylactic inoculation Nil	Slight	1	100-00	1	100-00	—	—	—	—	—	—	—	—	11-00	—	—	11-00
	Medium	3	100-00	3	100-00	—	—	—	—	—	—	—	—	24-33	—	—	24-33
	Bad	8	7	87-50	7	87-50	—	—	—	—	1	12-50	—	24-57	—	43-00	26-88
	Very bad	9	4	44-44	4	44-44	—	—	—	—	5	55-56	—	38-50	—	36-80	37-55
	Hopeless	1	—	—	—	—	—	—	—	—	1	100-00	—	—	—	35-00	35-00
Total ...		22	15	68-18	15	68-18	—	—	—	—	7	31-82	27-33	—	—	37-43	30-55
<i>Effectives</i> Propylactic inoculation 1 c.c.	Slight	1	100-00	1	100-00	—	—	—	—	—	—	—	—	10-00	—	—	10-00
	Medium	11	11	100-00	11	100-00	—	—	—	—	—	—	—	20-36	—	—	20-36
	Bad	8	7	87-50	7	87-50	—	—	—	—	1	12-50	—	22-14	—	30-00	23-13
	Very bad	12	9	75-00	7	58-33	2	16-67	—	—	3	25-00	23-71	68-50	28-67	32-42	32-42
	Hopeless	2	—	—	—	—	—	—	—	—	2	100-00	—	—	47-50	47-50	47-50
Total ...		34	28	82-35	26	76-47	2	5-88	—	—	6	17-65	21-35	68-50	35-17	26-56	26-56
<i>Effectives</i> Propylactic inoculation 4 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	2	1	50-00	1	50-00	—	—	—	—	1	50-00	19-00	—	—	75-00	47-00
	Hopeless	1	—	—	—	—	—	—	—	—	1	100-00	—	—	—	50-00	50-00
Total ...		3	1	33-33	1	33-33	—	—	—	—	2	66-67	19-00	—	62-50	48-00	48-00
<i>Invalids</i> Propylactic inoculation Nil	Slight	2	2	100-00	2	100-00	—	—	—	—	—	—	—	17-00	—	—	17-00
	Medium	3	3	100-00	1	33-33	2	66-67	—	—	—	—	—	19-00	21-50	—	20-67
	Bad	6	2	33-33	2	33-33	—	—	—	—	4	66-67	13-50	—	—	76-75	53-67
	Very bad	15	3	20-00	1	6-67	2	13-33	—	—	12	80-00	14-00	32-00	—	32-17	30-93
	Hopeless	9	—	—	—	—	—	—	—	—	9	100-00	—	—	—	7-78	7-78
Total ...		35	10	28-57	6	17-14	4	11-43	25	71-43	25	71-43	15-67	26-75	30-32	27-54	27-54
<i>Invalids</i> Propylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	3	100-00	2	66-67	1	33-33	—	—	—	—	—	18-00	28-00	—	21-33
	Bad	10	6	60-00	5	50-00	1	10-00	—	—	4	40-00	32-60	78-00	63-25	49-40	49-40
	Very bad	16	6	37-50	6	37-50	—	—	—	—	10	62-50	53-87	—	19-20	32-19	32-19
	Hopeless	7	—	—	—	—	—	—	—	—	7	100-00	—	—	11-14	11-14	11-14
Total ...		36	15	41-67	13	36-11	2	5-56	21	58-33	21	58-33	40-15	53-00	24-90	31-97	31-97



Table XII.

GROUP B IX. Initial dose. 1500 million. (Dose doubled every three days.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total		To duty		Invalided		Deaths		Duty		Invalided	Died	All cases	
		No.	%	No.	%	No.	%	No.	%	No.	%				
<i>Effectives</i> Prophylactic inoculation Nil	Slight	7	100-00	7	100-00	—	—	—	—	—	—	18-14	—	18-14	—
	Medium	31	100-00	30	96-77	1	3-23	—	—	14-73	31-00	—	—	15-26	—
	Bad	67	61	91-04	52	77-61	9	13-43	6	8-96	22-62	37-56	21-50	24-52	—
	Very bad	24	14	58-33	6	25-00	8	33-33	10	41-67	26-33	30-37	14-80	22-87	—
	Hopeless	2	—	—	—	—	—	2	100-00	—	—	—	6-50	6-50	—
Total ...		131	113	86-26	95	72-52	18	13-74	18	13-74	20-03	34-00	16-11	21-41	—
<i>Effectives</i> Prophylactic inoculation 4 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	100-00	2	66-67	1	33-33	—	—	21-50	—	—	—	29-00	—
	Bad	5	100-00	5	100-00	—	—	—	—	23-80	—	—	—	23-80	—
	Very bad	1	100-00	1	100-00	—	—	—	—	41-00	—	—	—	41-00	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		9	9	100-00	8	88-89	1	11-11	—	25-37	—	44-00	—	27-44	—
<i>Invalids</i> Prophylactic inoculation Nil	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	100-00	—	—	3	100-00	—	—	—	—	23-00	—	23-00	—
	Bad	10	9	90-00	1	10-00	8	80-00	1	10-00	24-00	36-87	47-00	29-20	—
	Very bad	11	8	72-73	—	—	8	72-73	3	27-27	—	25-50	16-00	22-91	—
	Hopeless	2	—	—	—	—	—	2	100-00	—	—	—	11-00	11-00	—
Total ...		26	20	76-92	1	3-85	19	73-07	6	23-08	24-00	26-00	19-50	24-42	—
<i>Invalids</i> Prophylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Very bad	1	100-00	—	—	1	100-00	—	—	—	—	15-00	—	15-00	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total ...		1	1	100-00	—	—	1	100-00	—	—	—	15-00	—	15-00	—
<i>Invalids</i> Prophylactic inoculation 4 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	7	7	100-00	—	—	7	100-00	—	—	—	24-57	—	24-57	—
	Very bad	9	4	44-44	—	—	4	44-44	5	55-56	—	38-75	14-20	25-11	—
	Hopeless	2	—	—	—	—	—	2	100-00	—	—	—	7-50	7-50	—
Total ...		19	11	57-89	—	—	11	57-89	7	34-89	—	90-73	12-99	99-94	—

Table XIII.  
(GROUP B x. Initial dose, 12,000 million. (Repeated weekly.)

Class	Character of case	Recoveries										Average number of days in hospital			
		Total cases	Total		To duty		Invalided		Deaths		Duty				Invalided
			No.	%	No.	%	No.	%	No.	%		No.	%		
<i>Effectives</i> Prophylactic inoculation 1 c.c.	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bad	1	—	—	—	—	—	—	—	1	100-00	—	—	23-00	23-00
	Very bad	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Hopeless	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total ...	1	—	—	—	—	—	—	—	1	100-00	—	—	23-00	23-00

Table XIV.  
Division Y. Summary of Tables VII to XIII.

Class	Character of case	Recoveries										Average number of days in hospital							
		Total		To duty		Invalided		Deaths		Duty						Invalided		Died	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>Effectives</i> Propylactic inoculation Nil	Slight	19	19	100-00	18	94-74	1	5-26	—	—	—	—	—	—	13-83	10-00	—	—	13-63
	Medium	37	57	100-00	55	96-49	2	3-51	—	—	—	—	—	—	13-78	21-00	—	—	14-03
	Bad	97	89	91-75	79	81-44	10	10-31	8	8-25	—	—	—	—	20-99	37-30	28-13	23-26	23-26
	Very bad	59	37	62-71	28	47-46	9	15-25	22	37-29	—	—	—	—	30-03	32-33	23-91	28-10	28-10
	Hopeless	9	—	—	—	—	—	—	9	100-00	—	—	—	—	—	—	31-00	31-00	31-00
	Total ...	241	202	83-82	180	74-69	22	9-13	39	16-18	—	—	—	—	19-44	32-55	26-41	21-79	21-79
<i>Effectives</i> Propylactic inoculation I c.c.	Slight	3	3	100-00	3	100-00	—	—	—	—	—	—	—	—	12-67	—	—	—	12-67
	Medium	27	26	96-29	26	96-29	—	—	1	3-71	—	—	—	—	16-27	—	18-00	16-33	16-33
	Bad	22	19	86-36	19	86-36	—	—	3	13-64	—	—	—	—	20-84	—	20-67	20-82	20-82
	Very bad	27	19	70-37	16	59-26	3	11-11	8	29-63	—	—	—	—	28-56	63-33	37-50	35-07	35-07
	Hopeless	5	—	—	—	—	—	—	5	100-00	—	—	—	—	—	—	26-20	26-20	26-20
	Total ...	84	67	79-76	64	76-19	3	3-57	17	20-24	20-53	63-33	30-06	23-99	23-99	23-99	23-99	23-99	23-99

Table XIV—*contd.*  
 Division Y. Summary of Tables VII to XIII.—*contd.*

Class	Character of case	Total cases	Recoveries						Deaths				Average number of days in hospital			
			Total		To duty				Invalided				Duty	Invalided	Died	All cases
			No.	%	No.	%	No.	%	No.	%	No.	%				
<i>Effectives</i>	Slight	3	3	100.00	3	100.00	—	—	—	—	—	—	10.67	—	—	10.67
	Medium	27	24	88.89	23	85.19	1	3.70	3	11.11	18.00	44.00	39.00	39.00	21.29	21.29
	Bad	56	49	87.50	47	83.93	2	3.57	7	12.50	22.74	86.00	20.57	24.73	24.73	24.73
	Very bad	56	39	69.64	37	66.07	2	3.57	17	30.36	29.05	83.00	35.65	33.52	33.52	33.52
	Hopeless 4 c.c.	8	—	—	—	—	—	—	8	100.00	—	—	21.87	—	21.87	21.87
Total ...		150	115	76.67	110	73.33	5	3.34	35	23.33	23.35	82.40	29.77	—	29.77	26.96
<i>Invalids</i>	Slight	10	8	80.00	5	50.00	3	30.00	2	20.00	11.40	9.33	9.50	—	—	10.40
	Medium	24	24	100.00	14	58.33	10	41.67	—	—	17.21	21.90	—	—	—	19.17
	Bad	37	28	75.68	16	43.24	12	32.44	9	24.32	34.00	37.83	52.67	39.78	52.67	39.78
	Very bad	47	19	40.43	6	12.77	13	27.66	28	59.57	30.00	26.08	28.04	27.74	28.04	27.74
	Hopeless Nil	19	—	—	—	—	—	—	19	100.00	—	—	16.89	—	16.89	16.89
Total ...		137	79	57.66	41	29.93	38	27.73	58	42.34	24.93	27.37	27.57	—	27.57	26.72
<i>Invalids</i>	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	7	5	71.43	3	42.86	2	28.57	2	28.57	22.00	51.50	15.50	28.57	15.50	28.57
	Bad	12	7	58.33	6	50.00	1	8.33	5	41.67	29.33	78.00	52.40	43.00	52.40	43.00
	Very bad	31	10	32.26	8	25.81	2	6.45	21	67.74	45.50	44.00	14.71	24.55	14.71	24.55
	Hopeless 1 c.c.	10	—	—	—	—	—	—	10	100.00	—	—	10.00	—	10.00	10.00
Total ...		60	22	36.67	17	28.33	5	8.34	38	63.33	35.65	53.80	18.47	—	18.47	26.28
<i>Invalids</i>	Slight	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Medium	3	3	100.00	3	100.00	—	—	—	—	35.33	—	—	—	—	35.33
	Bad	17	12	70.59	3	17.65	9	52.94	5	29.41	16.67	30.78	24.60	26.47	24.60	26.47
	Very bad	20	7	35.00	3	15.00	4	20.00	13	65.00	49.00	38.75	19.38	27.70	19.38	27.70
	Hopeless 4 c.c.	11	—	—	—	—	—	—	11	100.00	—	—	87.28	—	87.28	87.28
Total ...		51	22	43.14	9	17.65	13	25.49	29	56.86	33.67	33.23	16.24	—	16.24	23.65

Table XV.  
Summary of all Vaccine cases in Divisions X and Y.

Class	Character of case	Recoveries										Average number of days in hospital				
		Total		To duty		Invalided		Deaths								
		No.	%	No.	%	No.	%	No.	%	Duty	Invalided	Died	All cases			
Division X <i>Effectives</i>	Slight	7	100-00	6	85-71	1	14-29	—	—	—	8-33	9-00	—	8-43		
	Medium	10	100-00	6	60-00	4	40-00	—	—	—	13-67	16-50	—	14-80		
	Bad	16	14	87-50	9	56-25	5	30-25	2	12-50	17-00	25-40	12-00	19-00		
	Very bad	13	6	46-15	4	30-77	2	15-38	7	53-85	29-71	39-50	29-71	27-46		
	Hopeless	2	—	—	—	—	—	—	2	100-00	—	—	17-50	17-50		
Total all cases		48	37	77-08	25	52-08	12	25-00	11	22-92	14-20	23-42	24-27	18-77		
Total excluding "Hopeless" cases		46	37	80-43	25	54-35	12	26-08	9	19-57	14-20	23-42	25-78	18-83		
Division X <i>Invalids</i>	Slight	9	8	88-89	2	22-22	6	66-67	1	11-11	12-50	14-50	1-00	12-55		
	Medium	3	2	66-67	1	33-33	1	33-33	1	33-33	10-00	27-00	18-00	18-33		
	Bad	9	4	44-44	2	22-22	2	22-22	5	55-56	8-50	34-00	21-20	21-22		
	Very bad	10	4	40-00	—	—	4	40-00	6	60-00	—	—	78-25	19-83		
	Hopeless	6	—	—	—	—	—	—	6	100-00	—	—	19-17	19-17		
Total all cases		27	18	48-65	5	13-51	13	35-14	19	51-35	20-60	34-15	18-89	24-49		
Total excluding "Hopeless" cases		31	18	58-06	5	16-13	13	41-93	13	41-93	20-60	34-15	18-77	25-52		
Division Y <i>Effectives</i>	Slight	25	25	100-00	24	96-00	1	4-00	—	—	13-29	10-00	—	13-16		
	Medium	111	107	96-39	104	93-69	3	2-70	4	3-61	15-34	28-67	33-75	16-36		
	Bad	175	157	89-71	145	82-80	12	6-91	18	10-29	21-54	45-42	23-94	23-48		
	Very bad	142	95	66-90	81	57-04	14	8-86	47	33-10	29-29	48-36	30-47	31-56		
	Hopeless	22	—	—	—	—	—	—	22	100-00	—	—	26-59	26-59		
Total all cases		475	384	80-84	354	74-53	30	6-31	91	19-16	20-93	43-93	28-38	23-81		
Total excluding "Hopeless" cases		453	384	84-77	354	78-15	30	6-62	69	15-23	20-93	43-93	28-96	23-68		
Division Y <i>Invalids</i>	Slight	10	8	80-00	5	50-00	3	30-00	2	20-00	11-40	9-33	9-50	10-40		
	Medium	34	32	94-12	20	58-82	12	35-30	2	5-88	20-65	26-83	15-50	22-53		
	Bad	66	47	71-21	25	37-88	22	33-33	19	28-79	30-80	36-77	45-21	36-94		
	Very bad	98	36	36-73	17	17-35	19	19-38	62	63-27	40-65	30-63	21-71	26-72		
	Hopeless	40	—	—	—	—	—	—	40	100-00	—	—	12-93	12-93		
Total all cases		248	123	49-60	67	27-02	56	22-58	125	50-40	28-82	31-09	22-18	25-98		
Total excluding "Hopeless" cases		208	123	59-13	67	32-21	56	26-92	85	40-87	28-82	31-09	26-53	28-49		

Having therefore explained the method of classification used, we can turn to the other point, namely what conclusions can be drawn from the recorded results of these cases?

(1) In the first place we may note that, as far as the death rate is concerned, there is practically no difference in the results of vaccine treatment between cases which received prophylactic inoculation and those which did not, indeed if anything the cases which received a prophylactic inoculation did worse and had a higher death rate than those which received no such inoculation. The real difference between cases which received a prophylactic inoculation and those which did not, lies in the percentage of those returning to duty after the disease. Here the cases which received a prophylactic inoculation show a considerably higher proportion of men returning to duty than cases which received no prophylactic inoculation, among effective porters. The opposite is the case amongst invalids, but here a fallacy may possibly be introduced as it is still uncertain how long any immunity conferred by prophylactic inoculation lasts. It seems probable from statistics at present available, that the immunity, if any, only lasts for a comparatively short time, in which case invalids coming under the head of prophylactically inoculated, should really be classed among the non-inoculated.

(2) The only available records of cases not treated therapeutically with vaccine are of effective porters from which "hopeless" cases were excluded. On comparison of the death rate amongst these men, which was 31·38 per cent., with the cases treated with vaccine, we see that amongst effective porters treated with small doses of vaccine, the death rate was lowered to 22·92 per cent. whilst amongst a similar class of men treated with large doses of vaccine, it fell to 19·16 per cent. Here there is no room for any individual bias to enter, for all the vaccine cases were consecutive admissions to hospital whilst the control cases were to a large extent selected cases. If we exclude "hopeless" cases from the vaccine series, the death rate falls to 19·57 per cent. and 15·25 per cent. respectively. Therefore from these figures it may be concluded that the therapeutic use of the vaccine definitely influences the course of the disease for good and markedly reduces the death rate from bacillary dysentery when administered in sufficiently large doses.

In the case of invalids there are unfortunately no records of cases untreated with vaccine for comparison, and it is only possible from the recollection of the writer of the feeling of utter hopelessness in dealing with these cases in pre-vaccine days, to give an assurance that, poor as are the results shown for invalids under vaccine treatment, matters were far worse in the days when no vaccine was available. If the figures are thought to show an appallingly high death rate, it must be remembered that these invalids had been returned as useless to the forces in the field, that many of them were frightfully wasted from disease, and with no resistance left to protect them from the invasion of fresh hostile bacteria, and one may even look upon it as a triumph of medicine that, of their number, we were able to send back to the front even as small a

proportion as 13·51 per cent. in one set of cases and 27·02 per cent. in the other.

It will be noted that the statistics show improvement in the death rate with vaccine, not only amongst the milder cases, but also amongst the "very bad" or toxæmic class.

Besides comparison of the death rate, it is interesting to compare the statistics showing the percentage of cases returned to duty, a matter of great importance in a campaign when every effective porter was an asset to our forces. In pre-vaccine days it was considered that, if a man went into hospital suffering from dysentery, it was almost a certainty that, if he escaped death, he would be sent home as an invalid, only 21·32 per cent. being returned to duty. Compare with this the figures of effective porters treated with vaccine. Even with small doses, inadequate as they seemed in the light of later experience, over 50 per cent. of cases of dysentery were returned for further service at a time when the standard of fitness for duty was very high, and with the larger doses almost 75 per cent. of treated cases were sent back to duty. As has been mentioned above, a small proportion from 13·51 per cent. up to 27·03 per cent. of men returned as invalids and admitted to hospital with dysentery, were saved for a further period of usefulness in the field, a rather larger proportion than were returned to duty from the pre-vaccine series of selected effectives. From the point of view therefore of military efficiency, the vaccine was an important asset in the medical armamentarium.

In the statistics of treated cases, the average number of days spent in hospital has been included. Apparently the vaccine has very little effect on the number of days cases remained under treatment, though the average time in the hospital of vaccine cases was undoubtedly increased by the longer time cases, which eventually died, remained in hospital. In the pre-vaccine series of porters, similar cases died in a much shorter time.

(3) As regards the dosage of vaccine advisable, full statistics of each different dosage tried are included in the Tables. Certain of these were used with too small a number of cases to be useful for any statistical purpose, but on consideration of the various groups, when a sufficient number of cases are included to make conclusions reliable, it would seem that the results obtained with a dosage of 2 c.c. (3000 million), 4 c.c. and 8 c.c. vaccine on the first, fourth and eighth days were the most satisfactory and this is the dosage which is now recommended for general use.

(4) A small series of cases was tried with autogeneus dysentery vaccines, but the numbers were too few to admit of statistical use. The general impression gathered, however, was that there was no advantage in their use over the polyvalent dysentery vaccine.

From the above facts it is recommended that the treatment of dysentery cases should be carried out on the following lines:

(1) Eliminate amoebic, bilharzial, and malarial dysentery by microscopical examination.

(2) Put the patient to bed.

(3) Give an aperient, and keep the bowels open by the use of magnesium sulphate in small doses three or more times a day.

(4) Administer *on the first day* or at the first possible moment, an initial dose of 2 c.c. (3000 million) of the polyvalent anti-dysenteric vaccine, followed by 4 c.c. on the fourth day and 8 c.c. on the eighth day, if necessary.

(5) Keep the patient on a light diet till the stools are normal in appearance and semi-solid, then gradually change to normal diet, being guided by the appearance of the stools.

(6) In the event of intractable diarrhoea setting in, give *B. coli communis* vaccine in  $\frac{1}{2}$  c.c., 1 c.c., and 2 c.c. doses at intervals of 3 days subcutaneously and by the mouth Pulvis Rhei and sodii bicarbonas one drachm of each thrice daily till the stools become normal in colour and tenesmus disappears.

It may be mentioned that, as post dysenteric diarrhoea was so common, and prolonged treatment to such an extent, an attempt was made to combine the treatments with anti-dysenteric vaccine and *B. coli* vaccine by giving them simultaneously, but no advantage was gained, the *B. coli* failing to produce the same effect as it does when it follows anti-dysenteric vaccine.

It must be noted that certain cases clinically and microscopically of bacillary type failed to respond at all to the anti-dysenteric vaccine. An explanation of this class of case may possibly be that heterologous dysentery bacilli were the causal organisms, or again the case may have been due to *Entamoeba histolytica* originally and treated as such, but secondary organisms, such as *B. coli communis*, may have got implanted on the healing amoebic ulceration, and have kept up the dysenteric condition. Certainly some of these cases responded markedly to a *B. coli* vaccine.

It is also worth recording that the vaccine in certain patients produced an immediate effect, the stools diminishing rapidly in number, their character improving, and the general condition of the patient becoming much better. It has happened too often to be merely coincidence, and it must be concluded therefore that one of its effects may be the immediate stimulation of phagocytosis. Independent testimony to this and also to the value of the vaccine as a therapeutic agent has been received from other observers from whose reports the following statements are quoted.

Capt. Shircore, E.A.M.S., Native Civil Hospital, Mombasa: "In my experience the best results obtained with anti-dysenteric vaccine are in chronic dysenteries. Its effects are immediate, *i.e.* within 18 to 24 hours, and whatever may be the explanation of such rapid action, nevertheless this result can be observed clinically. I regard it as a valuable therapeutic agent and would use it without fail in the type of cases mentioned in preference to any other line of treatment that I know of; and with confidence that if it was going to do good at all, it would do so promptly."

Capt. Mackinnon, E.A.M.S., K.A.R. Hospital, M'bagathi: "Although the preparation is a vaccine, I have usually found that there is an immediate

marked improvement after the first dose in nearly all cases in which a cure is obtained. There is usually a marked reaction and the temperature may run up to 103° F. or even more. I have noticed in several cases that, on the day following the inoculation, patients who have been passing 15 to 20 motions a day are suddenly reduced to one, or even to none, and show no signs of relapse thereafter."

Other observers have also noted a similar result with anti-dysenteric vaccine. See Skalski and Sterling (1917, *Deutsche med. Wochenschr.* XLIII. 713, abstract in *Trop. Dis. Bull.* x. 140), and Margolis (1917, *Deutsche med. Wochenschr.* XLIII. 783, abstract in *Bull. Inst. Pasteur*, xv. 557).

A testimony to the efficacy of the vaccine in treatment was given to the writer by two of his hospital dressers, who contracted dysentery while on duty, and insisted on being treated with the vaccine, although as a rule a native is distinctly averse to having needles thrust into him.

As a fact which requires further investigation, the writer wishes to record a general constitutional effect of the anti-dysenteric vaccine. A patient who had been in hospital for some considerable time with a bad ulcer of the foot, and a chronic inflammatory condition of the calf of the leg probably resulting therefrom, contracted dysentery. He was treated with anti-dysenteric vaccine in the usual way, and not only rapidly recovered from the intestinal disease, but the ulcer and the inflamed leg also became cured although they had resisted all other forms of treatment for months. So marked was the effect, that a case with a similar condition of ulcer and chronic cellulitis of the leg was tentatively given a dose of anti-dysenteric vaccine, and immediately cleared up. Unfortunately no other cases with exactly similar lesions have occurred, and no smear was taken from the ulcers of these two patients. The results with the vaccine are suggestive, however, and it has yet to be investigated whether dysentery bacilli do or do not occur in similar ulcers of the skin.

In conclusion the writer's thanks are due to Dr P. H. Ross, Director of Laboratories, E.A.P., whose suggestion to sterilise the vaccine with carbolic acid instead of heat made it possible to use large doses in treatment, to Dr J. Harvey Pirie, Institute of Medical Research, Johannesburg, from whose work on the dysentery bacilli of E. Africa, the idea of vaccine treatment was first formulated by the writer, and to both these officials for the way in which, under great difficulties and stress of other work, they so maintained the supply of the vaccine as to meet even excessive calls on it. The writer has also to thank Capt. J. A. M. Clarke, R.A.M.C., who supplied the records of cases detailed in Group B IX, and to Colonel Clemesha, D.D.M.S., E.A.E.F., for permission to publish this paper.



## CATS AND HUMAN DIPHTHERIA.

By WILLIAM G. SAVAGE, M.D.,  
*County Medical Officer of Health, Somerset.*

It is a widely accepted belief amongst medical men, particularly those who are Medical Officers of Health, that cats may suffer from diphtheria and convey it to human cases and that they are a not uncommon source of infection. It is of considerable practical importance to determine to what extent this belief is based upon reliable scientific data or whether it is another of those opinions, still far too prevalent, which arise from the acceptance of insufficiently tested and incorrectly interpreted observations.

In dealing with this subject I propose to give first a brief account of the available evidence upon which the association of cats with diphtheria has been built, then a summary of my own investigations and lastly a critical consideration of the whole of the data available.

### EVIDENCE ASSOCIATING CATS WITH HUMAN DIPHTHERIA.

This is both experimental and epidemiological.

Of the experimental work the most considerable in volume and of most importance as regards its influence upon contemporary and later medical opinion is the work of Klein (1888, 1889, 1890). This investigator *inoculated* eight cats subcutaneously and two intravenously with pure cultures of diphtheria bacilli. Four were unaffected but the other six showed swelling at the site of inoculation followed by death after five to eleven days. Postmortem all the fatal cases showed extensive hæmorrhagic oedema locally and certain special kidney changes characterised by enlargement of the whole organ, almost the entire cortex being grey and fattily degenerated, the medulla appearing by contrast much congested. Microscopically the uriniferous tubules were swollen and the epithelium showed extensive fatty degeneration or broken down into a granular debris. Klein describes this condition of the kidney as extremely characteristic of diphtheria disease in the cat. The condition of the supra-renal capsules is not mentioned.

He also caused death or paralysis in two cats by injecting diphtheria bacilli direct into the trachea by a syringe pushed through the anterior wall.

One kitten and two cats fed with agar cultures in milk remained well, but two other cats fed on three separate occasions became very thin, although showing no other symptoms, and were killed three and four weeks after the start of the experiment. Both showed livers much enlarged, in one the kidneys

were large with white cortex, in the other the kidneys were not conspicuously large but their cortex was pale with a few whitish patches. Not the slightest bacteriological proof is given or evidence supplied of the presence of any clinical or pathological features of diphtheria but Klein remarks "From these experiments it is seen that by *repeated* feeding with cultures of diphtheria bacilli distributed in milk, unquestionable diphtheria disease can be produced in the cat." Klein also laid stress upon certain cases of accidentally acquired diphtheria (so called) in cats. The symptoms were an acute catarrhal affection of the conjunctiva and of the respiratory passages. The diagnosis of diphtheria was based upon the pathological appearances of the kidney and the appearance in one of the cats of a grey membrane covering the lower part of the larynx and upper part of the trachea in which "the microscope showed crowds of diphtheria bacilli as smaller or larger groups in the necrotic mucous membranes." Diphtheria bacilli were also demonstrated in the bronchial and tracheal exudation of another cat. Nine months later a somewhat similar outbreak occurred with similar pathological lesions and the presence of bacilli morphologically like diphtheria bacilli.

Klein records his examination of three cats sent to him from different sources and all associated with human cases of diphtheria. All three cats had been ill. The kidney condition which Klein records as typical of diphtheria was found in each case but no diphtheria bacilli were cultivated. On the basis of the pathological appearances and their likeness to the conditions induced in cats by inoculation with Klebs-Löffler bacilli Klein considered these were cases of cat diphtheria.

Renshaw (1885) in 1874 administered diphtheric membrane from human cases to a number of cats. Most died after illness and Renshaw reported finding diphtheritic membrane lining the fauces, bronchial tubes, etc. No evidence was adduced that the lesions were due to diphtheria bacilli and not associated with pathogenic cocci, etc.

Welch and Abbott (1891) inoculated into the trachea of a half-grown kitten a platinum loopful of a pure culture of the diphtheria bacillus. The animal died on the third day with the production of a diphtheritic membrane, containing diphtheria bacilli, in the trachea and larynx. No other noticeable lesion was found "unless it be a greater degree of fatty metamorphosis of the renal epithelium than is normal in kittens."

The above constitutes the only experimental work I have been able to trace.

Evidence is also available on the epidemiological side in the form of instances in which it is reported that the disease has been transmitted to human cases through the agency of cats. Without making a thorough search of the literature I have collected particulars of a number of such cases.

Turner (1886) appears to have been the first to report the association of illness amongst cats with cases of human diphtheria and he drew attention to their presence in a number of small outbreaks. The evidence was entirely

circumstantial and consisted of the concurrent presence of human cases with cats ill with such symptoms as swelling of the neck, foul discharge from the nostrils, eye inflammation, etc.

Bruce Low (1888) reported a similar relationship in an outbreak at Enfield.

Williams (1895) reported that in the same house as a case of human diphtheria three cats were taken ill with wasting, loss of appetite, inability to swallow, cough and expectoration and all three died. One cat was sent to Klein for examination who reported that there was extensive disease of the lung not uncommon amongst cats.

Gwynn (1893) reported at Hampstead the association of a case of human diphtheria with an ailing cat suffering from a bad swollen throat. Later it showed an unhealed abscess in the vicinity of the throat. No bacteriological examinations were made.

Dowson (1895) examined a cat which became ill in a house in which a child had died 14 days earlier from diphtheria. Cultures from the lung gave a pure culture of an organism morphologically resembling the diphtheria bacillus which was not further investigated.

Symes (1896) mentions a kitten which was in close and constant contact with a fatal case of diphtheria and which subsequently was attacked with vomiting and diarrhoea with slight swelling of the neck and with patches of yellow exudation on the fauces. These patches extended, the cat became much emaciated and died. Unfortunately the cat was buried before bacteriological examinations could be made.

Barras (1905) reports that in two cases of diphtheria in Govan a history of illness affecting the cats of the families attacked was ascertained. The throats of two of these cats were examined in the laboratory and micro-organisms were cultivated on artificial media which were found to correspond in every respect to those of the human diphtheria bacillus. No particulars are given of the tests employed for the identification nor are any details furnished as to the degree or nature of the illness from which the cats suffered. Barras also mentions another cat in Govan—a stray cat—which had obtained a home in one of the families affected and which was found to be suffering from post-diphtheritic paralysis of the legs. Apparently no bacteriological examinations were made.

Porter (1908) records a number of cases of diphtheria in a household containing two petted and favourite cats. The cats were not ill. Swabs were rubbed over the fur of each cat. One yielded negative results but smears from the culture from the other cat showed a considerable number of organisms identical in appearance with the Klebs-Löffler bacillus both when stained by methylene blue and by Neisser's method. Porter was unable to isolate the organism in pure culture.

Mapleton (1913), in connection with an outbreak of diphtheria amongst the children living in the Cottage Home, Newton Abbot, reported that there were "three cats in this Home in somewhat intimate association with the chil-

drugs and a bacteriological examination of these proved that two of them were infected." Further inquiries showed that the examinations made were ordinary swabbings grown on blood serum and that the bacilli were not isolated in pure culture nor were any animal inoculations made. In this case the cats were not definitely ill although one was obviously not in health and were not suspected of having conveyed any infection. They were examined to prevent the possibility of their carrying infection elsewhere.

Webb (1914) reported an interesting outbreak at Leigh (Lancashire) amongst cats to which his attention was directed in connection with a case of diphtheria in a child. In the house with this human case the cat had been ailing for more than two weeks, had a peculiar cough, could not miaow properly, refused food, was very thin and could hardly move. During the previous week a cat next door had died presenting the same symptoms and a cat across the way had also died with the same symptoms.

The throat of the cat in the house with the diphtheria case was in a dirty sloughy condition and cultivations showed "a bacillus having the characteristics of the Klebs-Löffler bacillus." The organism was not isolated in pure culture and no cultural or inoculation tests were carried out. Webb states that five other cats in the street were found to be ailing and were destroyed.

Priestley (1915) investigating a number of cases of diphtheria at the Stockwell Orphanage found nine cats in that institution. All were bacteriologically examined although none of them were apparently ill. He reports "three of them in the boys' department and one belonging to the girls' department carried the diphtheria bacillus." In a personal communication he was kind enough to inform me that the bacilli were morphologically and culturally undistinguishable from the true Klebs-Löffler bacillus. No inoculation experiments were carried out nor is any information available as to what cultural tests were employed but apparently they were not isolated in pure culture and then tested.

The significance of the above data will be considered after my own experiments have been recorded.

#### THE AUTHOR'S INVESTIGATIONS.

These have been along three lines of inquiry:

- A. The bacteriological examination of the throat and nose of healthy cats not associated with any cases of human diphtheria.
- B. The bacteriological and pathological examination of cats associated with human cases of diphtheria.
- C. Experimental investigations with kittens.

##### A. EXAMINATION OF HEALTHY CATS.

Eight cats and 12 kittens were examined, the throat being examined in every case while for most of the kittens and for a few of the cats the nose was also swabbed. One cat was examined twice. The method of examining

consisted in taking one or more swabs in the ordinary way and then charging at least three blood serum tubes and one agar tube in series without recharging. The usual order was 1 serum, 1 agar, 1 serum, 1 serum tube, the object being to obtain considerable dilution of bacteria on the last two serum tubes so that discrete colonies could be obtained. In some cases pea-flour trypt-agar plates, a medium upon which diphtheria bacilli grow well, were also inoculated. All the 12 kittens failed to show any bacilli which at all resembled diphtheria bacilli.

The eight cats were very different. Swabs from three of them showed no bacilli which might be taken for Klebs-Löffler bacilli. The results from the other five were as follows. The characters refer to films stained by methylene blue.

(1) Short bacilli, well marked beading chiefly at ends but some with central staining. Some curved and longer forms very closely resembling Klebs-Löffler bacilli. Bacillus isolated in pure culture.

(2) A number of bacilli present closely resembling Klebs-Löffler bacilli, thin, curved some thicker at ends and a few, but not the majority, showing polar staining. Do not show granules when treated with acetic acid. Isolated in pure culture.

(3) A large number of bacilli present resembling Klebs-Löffler bacilli. Curved, beaded but not definitely clubbed. No dark blue granules when treated with acetic acid. A bacillus resembling this organism isolated in pure culture.

(4) Numerous bacilli present which are curved, thin and beaded. Not thickened at ends but closely simulate Klebs-Löffler bacilli. Isolated in pure culture.

(5) A very mixed growth but contains a number of bacilli which very closely resemble true diphtheria bacilli being long, curved bacilli with granules at end but not in the middle. Granules show up well when the films were treated with acetic acid. Differ from typical Klebs-Löffler bacilli in being rather more uniform in size and the absence of clubbing. Was unable to isolate in pure culture.

No. 2 is of particular interest as it was the cat in a large girls' school. This cat was re-examined exactly three months later. = 2 *a*.

(2 *a*) Many bacilli closely resembling Klebs-Löffler bacilli present, being curved, and granular, thicker at one end and showing dark blue granules when the film was treated with acetic acid. Isolated in pure culture.

The bacilli isolated from cats (1), (2) and (3) were all alike so far as they were culturally investigated. They morphologically resembled true diphtheria bacilli less when isolated in pure culture although still superficially resembling it. Culturally they were distinct, the agar and blood serum growths being definitely yellow or yellow-white in colour while they produced no acid in glucose media and in litmus milk produced definite alkali after four to five days' growth.

The bacillus from cat (2 *a*) was extremely like the true diphtheria bacillus

both morphologically and culturally. It produced acid in glucose broth and grew like the diphtheria bacillus in milk. It was only distinguished so far as it was tested by the blood serum colonies having a distinct yellow tinge. Morphologically it was indistinguishable. It was non-pathogenic to a guinea pig.

It may be added that the films from the serum cultures made direct from the swabs were shown to the county bacteriologist in the laboratory who was examining several thousand suspected diphtheria swabs every year and he as well as myself was unable to distinguish the films from those from swabs from human throats containing true diphtheria bacilli.

We have therefore the striking fact that two experienced bacteriologists were unable to distinguish with any certainty these bacilli in mixed smears from true diphtheria bacilli although in several instances we were in some doubt and could not pronounce them typical bacilli since minute differences were apparent. In several cases the resemblance was extremely close. All of them however (with the possible exception of the strain from cat (2 a)) were definitely not true diphtheria bacilli. Present in 66 per cent. of the adult cats it is interesting to note their entire absence from all 12 kittens.

#### B. EXAMINATION OF CATS ASSOCIATED WITH HUMAN CASES.

Although through *Public Health* medical officers of health were specially asked to send me such cases, none reached me in this way and the few cases investigated were all obtained through my personal efforts. Four were investigated in 1916 and one in 1919.

*Case 1.* Case of severe diphtheria in a house. A favourite cat much in the room with this case and with two children who suffered from severe colds but whose throats showed no diphtheria bacilli when swabbed.

The cat showed no signs of sore throat or other illness. Swabs from its throat were implanted on several blood serum tubes in series. The films showed numerous bacilli which in the primary films could not be distinguished from true diphtheria bacilli either by myself or the laboratory bacteriologist. A short form with marked polar staining. Isolated in pure culture the bacillus decidedly less closely resembled the Klebs-Löffler bacillus morphologically, while the yellow colonies and absence of acid production in glucose clearly showed it was not that organism.

If morphological appearances from the mixed growth swab had been relied upon undoubtedly this cat would have been classed as infected with diphtheria bacilli and would probably have been credited as the source of the infection.

*Case 2.* A human diphtheria case (a woman of 21) in a house. The house cat came into close association with this woman who was very fond of the animal. The cat was said to have been ailing for some days before the case was notified but no very clear particulars as to symptoms were forthcoming.

The cat when examined showed no local or general symptoms and the bacteriological examination of the throat swab showed a few bacteria suspiciously like diphtheria bacilli but nothing more definite.

*Case 3.* The cat in the house was closely associated with a case of human diphtheria. The animal remained throughout quite well and the cultures from the swab from its throat showed no bacilli at all like true Klebs-Löffler bacilli.

*Case 4.* A boy of eight years developed diphtheria on Nov. 22nd, 1916. The house cat was said to be "off its food" for about a week before that date and subsequently showed difficulty of swallowing as if it had a sore throat. The patient was very fond of this cat and fondled and nursed her regularly.

The cat was swabbed Dec. 6th. The films made from the blood serum cultures showed a few clusters of bacilli which morphologically rather resembled diphtheria bacilli of the short type. They were slightly curved, beaded and thicker at one end but rather thick compared with the true organism. Impossible to exclude as not Klebs-Löffler bacilli. They were only present in very small numbers and could not be isolated in pure culture. I was fortunate in being able to secure the cat and re-swabbed it in the laboratory but the cultures from this swab failed to show any bacilli suspicious of Klebs-Löffler bacilli.

The cat was killed and a postmortem examination made. There were no pathological lesions, the kidneys and other organs being quite healthy. Further swabs from the back of the throat and from the upper part of the trachea showed culturally no bacilli resembling diphtheria bacilli.

It may be mentioned that the cat when the first swab was taken and when later on examined at the laboratory showed no symptoms at all of ill health.

*Case 5.* A child, aged five, was removed to the isolation hospital suffering from diphtheria the diagnosis being bacteriologically confirmed. The child was said to have been ailing for at least ten days before removal. An elder sister (16 years) also had a sore throat before the notified case and was possibly the source of infection, but when swabbed the day the notified case was removed to hospital showed no diphtheria bacilli. According to the mother of the case the house cat was ill for 3-4 weeks before the child of five was notified, refusing to eat and with some discharge from the nostrils and mouth. The cat however gave birth to some kittens soon afterwards and then recovered and was quite well at the time of the removal of the case to hospital. The cat was brought to the laboratory April 16th, i.e. six days after the removal to hospital of the case, and swabbed. It showed no signs of any illness or nasal discharge. The animal was killed and postmortem showed no membrane in the throat or any lesions anywhere.

The films from the blood serum cultures made showed (mixed with abundant cocci) bacilli which were beaded, curved and thicker at one end and which could not be distinguished from diphtheria bacilli. With great difficulty this organism was isolated in pure culture and investigated. Morphologically

it could not be distinguished from the diphtheria bacillus and the blue granules after treatment with acetic acid were very marked. Culturally it grew like diphtheria bacilli on ordinary agar and blood serum but unlike our ordinary diphtheria strains more abundantly on the former than on the latter. Indeed it did not grow well on blood serum. It produced acid in glucose broth but none in lactose or mannite broth while it produced a trace of acid in saccharose broth.

To test its pathogenicity a guinea pig was inoculated subcutaneously from a broth culture reinforced by the growth on a tube of pea-flour trypt-agar (*i.e.* a very heavy dose) but the animal was unaffected.

The cultural characters agree with the diphtheria bacillus for the most part and this may possibly have been a non-pathogenic Klebs-Löffler bacillus.

### C. EXPERIMENTAL INVESTIGATIONS WITH KITTENS.

If, as has been so often asserted, cats suffer from diphtheria or even if it be merely advanced that they act as carriers of the diphtheria bacillus in their throat or nose, it should be possible to infect them artificially and set up either condition.

The possibility of this was tested in a long series of experiments. Kittens were used in preference to adult cats as both more easily handled and as likely to be more susceptible to infection than the grown up animals. In every case the throat and in nearly all cases the nose of the animals were swabbed before the experiments started to ascertain if diphtheria-like bacilli were present or absent.

The kittens were all kept in separate cages and very carefully examined for any signs of ill health. Weight observations were recorded but temperature testings were not found very helpful. The kittens were usually from four to six weeks old when the experiments started.

*Exp. 1.* Throat swabbed with, as far as possible, all the growth of a blood serum culture of *B. diphtheriae* transferred on a sterile swab. Immediately re-swabbed in the same way with a second blood serum culture of another strain.

Throat swabbed after three and seven days and several blood serum tubes inoculated. No diphtheria bacilli found. Animal showed no signs of any illness, quite lively and gained in weight. No local throat-lesions.

*Exp. 2.* Throat of same kitten re-swabbed a month after onset of *Exp. 1* with as far as possible the whole of a blood serum culture made direct from a swab taken from the throat of an acutely ill case of diphtheria. This culture showed about half the colonies *B. diphtheriae* and half streptococci and it was used as possibly the mixed growth might favour infection. Animal remained quite well and lively and a swab taken six days after the inoculation showed no diphtheria colonies on the several blood serum tubes inoculated.

Animal killed eight days after the second inoculation. Postmortem all the organs were healthy and sections of kidney and supra-renals showed no abnormalities. No trace of any lesions in the throat.

The virulence to guinea pigs of these three strains was not tested.



*Exp. 3.* Kitten about four weeks old fed on two consecutive days with milk containing the whole of two blood serum growths of two different strains of *B. diphtheriae*, one of which was fully virulent to a guinea pig. Animal showed no symptoms whatever and the throat swabbed six days after the first feeding yielded no diphtheria bacilli.

*Exp. 4.* Throat of the same kitten swabbed with, as far as possible, the whole of two blood serum cultures of *B. diphtheriae* (plus a small number of staphylococcus colonies) grown direct from the throat of an acute case of diphtheria. Throat swabbed seven days later showed no diphtheria bacilli. Animal showed steady increase in weight and no local or general symptoms.

Kitten killed 22 days after onset of the feeding and 15 days after the throat swabbing. No trace of membrane on throat, trachea or larynx. Internal organs normal and sterile. Sections of the kidney and supra-renals showed no pathological changes except some engorgement of the kidney blood vessels.

*Exp. 5.* Throat of a kitten about five weeks old swabbed with the whole of a blood serum growth from a fifth strain (strain B). This blood serum growth was direct from a swab from an acute case and showed *B. diphtheriae* in almost pure culture. Swabs from the throat taken 4 and 10 days after inoculation showed no diphtheria bacilli. The animal increased in weight and exhibited no signs of ill health.

*Exp. 6.* Thirteen days after *Exp. 5* started this kitten was injected subcutaneously with 2 c.c. of a five days old (trypsin pea-flour) broth pure culture of the same strain (strain B) of diphtheria bacillus. The animal was obviously ill within 24 hours of the injection and remained very quiet. The temperature which for the two days before the experiment had fluctuated between 99° and 100° rose to 104° F. the morning and also the evening after the inoculation. It remained between 102° and 104° for seven days, then rapidly dropped to below 100° C. and was 96° on the ninth day when the animal was moribund and was killed. The day before death very definite paralysis of the hind legs was observable. The postmortem examination showed no oedema or inflammation at the site of inoculation, no fluid excess in the thoracic cavity. The internal organs, apart from the kidneys and supra-renals, appeared healthy. Both kidneys were greatly enlarged and in section showed marked cloudy swelling of the cells of the cortex and medulla with enlargement of all the blood vessels. The supra-renals showed a condition of acute inflammation with dilated engorged blood vessels and swollen, turbid and indistinct cells in both cortex and medulla.

This experiment is important as showing that the same strain which was incapable of infecting or affecting the kitten by means of the throat was highly pathogenic when injected into the same animal.

*Exp. 7.* A rather older kitten (about two months) and weighing 830 grms. used for a combined throat swabbing and feeding experiment.

Throat swabbed with the whole of a blood serum culture of another

*B. diphtheriae* strain (strain J). Throat examined after two, five, and nine days showed no diphtheria bacilli.

Five days after the third inoculation fed with milk in which strain J had been grown for 24 hours fortified by the whole of a 24 hours growth on blood serum of the same organism.

Feeding with the same massive dose repeated on the following three days. Throughout the animal remained quite well and gained steadily in weight.

*Exp. 8.* The same kitten some weeks later injected subcutaneously with 1.8 c.c. of a two days glucose broth culture of strain J. The kitten showed a slight rise of temperature but no other symptoms and increased in weight. Killed 14 days after the injection showed no macroscopic or microscopic lesions. The growth in the broth culture was scanty and the kitten weighed when inoculated 1020 grms. so the dose was probably inadequate and this experiment hardly indicates the strain was non-virulent.

*Exp. 9.* The nose of a young kitten about four weeks old inoculated heavily with a pure culture on blood serum of *B. diphtheriae* (strain W) transferred on a thick blunt platinum needle. Previous swabbings of nose (both sides) and throat showed absence of any diphtheria bacilli-like organisms.

Nose swab examined 24 hours after the inoculation failed to show any diphtheria bacilli when grown on blood serum tubes. Both sides of the nose then again inoculated with *B. diphtheriae* in the same way as before and each nostril examined after 48 hours and eight days showed no diphtheria bacilli.

The animal remained perfectly well with no nasal discharge or any symptom.

*Exp. 10.* It may be argued that the failure to infect the kittens by the throat was due to the smooth healthy mucous membrane failing to afford a nidus for growth and the following and some other experiments were devised to deal with this objection.

Thirteen days after the onset of *Exp. 9* the throat of this kitten was well painted with a 20 per cent. solution of silver nitrate. Examined 24 hours later there was some reddening of the throat and superficial ulceration of the roof of the palate. Throat re-painted with the silver nitrate solution. After a further 24 hours there was definite redness with much mucous and some whitened areas on the palate. The throat was then inoculated with, as far as possible, the whole of a two days old blood serum culture of *B. diphtheriae* (strain W). Swabs from the throat 24 hours and 48 hours after the *B. diphtheriae* implantation when grown on blood serum showed no diphtheria organisms. The animal remained fairly well but was quiet and did not put on much weight but gradually recovered completely.

*Exp. 11.* Eight days after the start of *Exp. 10* this kitten was injected subcutaneously with 2 c.c. of a 24 hours broth culture (pea-flour trypt broth) of the same *B. diphtheriae* strain (strain W). Animal obviously ill 24 hours later. After 48 hours very ill, quiet and refused food. Three days about the same and after four days appeared better but died on fifth day.

Postmortem the animal showed the signs noted above as present in the other injected kitten except that the reddening of the supra-renals was trifling or absent. They showed however inflammation signs when sections were made and stained. The kidneys were especially engorged and enlarged. Exps. 10 and 11 show that this strain while highly pathogenic when injected was unable to establish itself upon the throat of the same animal even when a definite unhealthy and pathological nidus was provided.

*Exp. 12.* The throat of a young kitten swabbed with as far as possible the whole of a two days old blood serum growth of *B. diphtheriae* (the virulent strain W being used). Nose (left side) also inoculated from another serum culture of the same organism. No diphtheria bacilli found in the throat when examined after 24 and after 48 hours. Diphtheria bacilli found in the nose at the end of 24 hours but not after 48 hours or subsequently. Animal remained quite well.

*Exp. 13.* Nose and throat of another quite young kitten inoculated heavily from another *B. diphtheriae* strain (strain V). Both nose and throat were free from diphtheria bacilli when examined after 24 and 48 hours.

*Exp. 14.* This same kitten was then fed on eleven occasions over 14 days with a pea-flour broth culture of *B. diphtheriae* (strain W). This broth gave abundant growth and over this period as much as 100 c.c. of broth culture was administered. No illness or symptoms of any kind. The animal steadily increased in weight and the postmortem examination 3½ weeks after the commencement of the feeding showed no abnormalities.

*Exp. 15.* The hard palate (as far back as possible) of another young kitten was scarified by a scalpel making a number of superficial lesions. A *B. diphtheriae* (strain W) blood serum culture transferred on a sterile swab was then at once well rubbed into these superficial lesions. The animal remained quite well and examined 24 and 48 hours after showed no membrane or other lesions nor could diphtheria bacilli be cultivated from the swabs taken although a good many tubes were used.

*Exp. 16.* Throat of a young kitten swabbed with a strong solution of silver nitrate. Examined 24 hours after there was a well marked slough. The throat was inoculated with a blood serum culture of diphtheria bacilli. The animal was very ill and as it was in pain was killed. Here the throat treatment was too severe but swabs after 24 hours showed no diphtheria bacilli and postmortem there was no evidence of any true membrane although sloughs over the hard palate. Films from throat and trachea showed no bacilli like *B. diphtheriae*.

*Exp. 17.* The nose of another young kitten inoculated heavily with *B. diphtheriae* (strain W). Nose swabs taken previously showed no bacilli at all like diphtheria organisms. Nose examined after 24 hours and after 48 hours showed diphtheria bacilli colonies on the blood serum tubes and apparently more abundant after the longer period. None found after four and five days. Unfortunately not examined after three days. The animal

remained perfectly well, gained in weight, had no nasal discharge and post-mortem showed no lesions.

*Exp. 18.* Nose of another kitten inoculated heavily with two separate strains of *B. diphtheriae* (strains W and V).

After 24 hours diphtheria bacilli readily grown from the nose. Found but less readily after 48 hours. Examined after four days the bacilli were found in the cultures but with great difficulty and could not be found at all after five and six days. The kitten remained perfectly well throughout, increased in weight and showed no nose discharge or other local lesions.

*Exp. 19.* The nose of another kitten inoculated heavily with another *B. diphtheriae* strain (strain R) isolated a day or two previously from an acute case of diphtheria. After 24 hours a few diphtheria bacilli were grown from the nose but none after 48 hours, four and five days. Animal remained perfectly well without any local or general symptoms.

#### REMARKS ON THE EXPERIMENTS.

The results obtained with these young kittens are exceptionally uniform and concordant. It was found impossible to infect them by throat swabbing although very massive doses were invariably used, as many as 10 different strains employed and all of them were quite recently isolated from acute human cases of diphtheria. Further, four of the strains used were proved to be of high virulence and two definitely killed, when subcutaneously injected, young kittens they were unable to infect by natural channels of entry. The same failure to infect these animals with diphtheria or any local lesion was experienced when mixed cultures direct from human throats were used or when an artificial nidus for local growth was provided by chemical or mechanical means. Not only did the bacilli not infect but they failed to survive even and although such massive doses were inoculated they invariably disappeared after as short a period as 24 hours.

The implantations of vast numbers of diphtheria bacilli into the nasal cavities were equally ineffective in setting up any local or general lesions and (what I did not expect to find) were unable even to survive beyond a very short period. In only one experiment was there possible evidence of increase and only in one instance could the most diligent examination trace their persistence beyond four days.

The feeding experiments were also unsuccessful and the kittens consumed vast numbers of virulent diphtheria bacilli without the slightest evidence of harm.

These experiments so far from supporting the view that diphtheria is a naturally occurring disease of cats suggests that the secretion from the mucous membranes of the cat are peculiarly unfavourable to the growth of the diphtheria bacillus and will not even permit it to remain as a saprophyte.

## CRITICAL CONSIDERATION OF THE AVAILABLE FACTS.

There are two separate possibilities. On the one hand there is the suggestion that cats themselves may be affected with disease due to their infection with the diphtheria bacillus, and which therefore may be appropriately designated as cat diphtheria, while on the other hand we have to consider the possibility that these animals may act as carriers of diphtheria infection by harbouring the bacilli in their nose or throat or by their fur becoming infected.

As regards the first possibility it must be accepted from the work of Klein and others, corroborated by my own experiments, that cats are not immune to the toxins of *B. diphtheriae* and that when these are introduced under the skin or by other means such as direct injection into the trachea fatal results may occur with the development of pathological lesions not dissimilar from those which are observed when guinea pigs or other animals are similarly injected. This of course no more proves or even suggests that cats suffer from diphtheria than it does that guinea pigs so suffer or the latter animals from typhoid fever because they succumb to the injection of typhoid bacillus toxins.

A study of the experimental data in favour of cats suffering from diphtheria detailed in the first part of this paper shows that the evidence is extremely weak and unconvincing and does not warrant the interpretation placed upon it. My own considerable series of experiments, testing by many different ways the possibilities of natural infection, are in direct opposition to the widely accepted view that cats can suffer from a kind of diphtheria.

The evidence which has been adduced from the association of human diphtheria cases with cases of illness amongst cats whose throats are said to show diphtheria bacilli must be regarded as *valueless* in almost every case, for the following two reasons.

In all the instances that I have been able to find, with the exception of one case recorded by Barras and possibly Case 5 of my series, the diagnosis rests entirely upon the presence in the throat of the cats of bacilli morphologically like *B. diphtheriae*. Barras does not mention the steps he took to identify his bacillus and one cannot exclude the possibility of it being a pseudo-diphtheria bacillus. The results recorded above show that in *normal* cats no less than 66 per cent. showed bacilli which without isolation and study in pure culture I was unable to exclude as not true diphtheria bacilli. In my series of cats associated with cases of human diphtheria both Cases 1 and 4 would without doubt have been classed and accepted as proved cases of diphtheria in cats although a more complete investigation quite refuted this assumption.

The second reason is that the fact that the cat or cats were ill does not supply even *prima facie* evidence for suggesting that the human and cat conditions were related.

It is recognised by veterinary authorities that cats suffer from illness with symptoms which in some respects are analogous to those of human diphtheria but which are certainly not cases of diphtheria or due to *B. diphtheriae*.

Gray (1896), for example, states that the so-called diphtheria in the cat is a contagious disease of the cat, characterised by the presence of diphtheritic membranes on the fauces, pharynx or larynx and due to some micro-organism not yet determined. The disease has a mortality of quite 90 per cent.

Gofton (1913) says that the kidney condition described by Klein as pathognomic is an exceedingly common condition of the cat's kidney. He adds "affections presenting diphtheritic characters are met with in the cat and occur independently of human diphtheria, but the bacteriological investigation of these affections has up to the present always resulted in a failure to demonstrate the presence of the human bacillus." With a prevalent disease such as human diphtheria and a not very rare illness of cats with the above symptoms it is obvious that the two will be associated together in a certain proportion of cases. I investigated my first cases of supposed cat diphtheria (with negative results, not recorded here) just over 20 years ago and I have been on the look out for associated cases ever since and undoubtedly the association of the two conditions is infrequent. In fact we shall probably be correct in affirming that the two diseases are not associated together in any higher proportion than the relative frequency of the two conditions mathematically postulates. There are therefore no grounds for the assumption so commonly made that when the two conditions do happen to coincide we should assume a relationship of cause and effect between the two.

The interesting cases of illness in cats recorded by Webb (*loc. cit.*) are clearly examples of this infectious disease in cats and there is no justification for dragging in the human factor to account for them.

My experimental work affords no support to the suggestion that the cats may act as carriers by the bacilli living in their throat or nose. On the contrary it would appear that the mucous membranes of these animals are particularly inimical to these bacilli and that even under the most favourable circumstances they are unable to multiply or even survive for more than a trifling period. That the fur of these animals may be infected with these bacilli is of course quite a possibility and there is some experimental evidence in its favour (*e.g.* Remlinger, 1906) but in view of the low resistance powers of *B. diphtheriae* this is probably an unimportant factor in the spread of the disease.

Summing up the matter I am of opinion that the common and widely accepted view that cats can suffer from a naturally acquired disease caused by the diphtheria bacillus is entirely without foundation.

The reported cases of such an association are based upon insufficient examination and differentiation of the bacilli due to a failure to realise that a large proportion of healthy normal cats contain in their throats bacilli which closely resemble and are difficult to distinguish from the true *B. diphtheriae*.

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## A NOTE ON "DEFENCE RUPTURE" AND THE ACTION OF ELECTROLYTES.

By W. CRAMER AND W. E. GYE.

*Imperial Cancer Research Fund, London.*

THE etiology of tetanus and gas gangrene presents a biological problem of an unusual nature. When the organisms which cause these diseases are deprived of toxin, by washing in tap-water saline or by washing and then heating, they are non-pathogenic to laboratory animals. In a recent investigation of the problem<sup>1</sup> we have shown that soluble ionisable calcium salts enable detoxicated *B. tetani*, *B. Welchii*, *B. oedematiens* and *vibrion septique* to exert their pathogenic powers. We concluded that calcium owes this property to its local action on the tissues.

Our conclusions have been criticised by Shearer<sup>2</sup>, who offers an alternative explanation of the facts. Shearer has shown that the unbalanced Na ion rapidly kills the meningococcus, and has brought forward evidence to prove that the pneumococcus and *B. anthracis* lose their pathogenicity when treated with a solution of pure NaCl in distilled water. His explanation of our work is that by washing in saline the gas gangrene bacilli are robbed of their pathogenicity which is restored by adding Ca. According to Shearer our experiments are examples of the interaction of bivalent and monovalent ions on the normal stability of the cell wall. We think our previous papers contain sufficient evidence to nullify Shearer's view of our work. The following experiments show it to be incorrect.

### EXPERIMENT 1.

A large tube of meat broth was inoculated with spores of *vibrion septique* which had been kept in tap-water saline at room temperature for 15 months. In 24 hours there was a vigorous growth with evolution of gas. The culture was kept at 37° C. for 48 hours and was then washed twice in Ringer's solution<sup>3</sup>. The final thick deposit of particles of meat and spores was heated in vaccine phials to 80° C. for 25 minutes. On the 3rd December, equal quantities of the spores were added to (A) 5 c.c. of Ringer, and (B) 2.5 c.c. of Ringer to which 2.5 c.c. of a 1 per cent. solution of CaCl<sub>2</sub> was added. Ten mice were then inoculated with suspension (A) and ten with suspension (B).

<sup>1</sup> *Proc. Roy. Soc. Ser. 'b*, xc. 513; also 6th Scientific Report, Imperial Cancer Research Fund.

<sup>2</sup> *Journ. of Hygiene*, xviii. 337.

<sup>3</sup> Composition: NaCl 0.77 %; KCl 0.024 %; CaCl<sub>2</sub> 0.0208 %.



*Action of Electrolytes*

	Inoculum	Dose and site	Result
(A) Mice 1 to 10.	Suspension of v. s. spores in Ringer.	0.5 c.c. right flank.	4 Dec. All alive and well. The animals remained in good health. On 10 Dec. each was injected with 0.5 c.c. of 1% $\text{CaCl}_2$ ; 11 Dec. all were dead of gas gangrene.
(B) Mice 11 to 20.	Suspension of spores in equal quantities of Ringer and 1% $\text{CaCl}_2$ .	0.5 c.c. right flank.	4 Dec. 9 dead of gas gangrene; 1 alive and well. This animal remained in good health. On 10 Dec. it was injected with 0.5 c.c. of 1% $\text{CaCl}_2$ ; 11 Dec. dead of gas gangrene.

## EXPERIMENT 2.

4th December. A tube of serum agar was inoculated with spores of *vibrio septique* and the tube incubated anaerobically. 6th December a thick growth of a pure culture of *vibrio septique*. The culture was taken off from the surface of the serum agar by means of a platinum loop and was emulsified in 1 c.c. of Ringer; 0.5 c.c. of the emulsion was added to (A) 3 c.c. of Ringer and 0.5 c.c. to (B) a mixture of 1.5 c.c. of Ringer and 1.5 c.c. of 1 per cent.  $\text{CaCl}_2$ .

Six mice were inoculated with the suspension in Ringer and six animals with the suspension in Ringer +  $\text{CaCl}_2$ .

	Inoculum	Dose and site	Result
(A) Mice 1 to 6.	Suspension of organisms in Ringer.	0.5 c.c. right flank.	7 Dec. Five alive and well. One dead of gas gangrene. The five mice remained in good health. 10 Dec. Inoculated with 0.5 c.c. 1% $\text{CaCl}_2$ . 11 Dec. All dead of gas gangrene.
(B) Mice 7 to 12.	Suspension of organisms in Ringer + $\text{CaCl}_2$ .	0.5 c.c. right flank.	7 Dec. All dead of gas gangrene.

## EXPERIMENT 3.

4th December. A culture of *B. Welchii* in meat broth was made.

5th December. Vigorous growth. Culture pure. 50 c.c. of culture centrifuged and broth pipetted off from deposit, which was then washed thrice with sterile Ringer. The final deposit was emulsified in 1 c.c. Ringer; 0.5 c.c. of the emulsion was added (A) to 3 c.c. of Ringer; (B) 0.5 c.c. to a mixture of 1.5 c.c. Ringer and 1.5 c.c. 1 per cent.  $\text{CaCl}_2$ .

The following inoculations were made:—

	Inoculum	Dose and site	Result
(A) Mice 1 to 6.	Suspension of <i>B. Welchii</i> in Ringer.	0.5 c.c. right flank.	All alive and well; the mice have remained in good health.
(B) Mice 7 to 12.	Suspension of <i>B. Welchii</i> in Ringer and $\text{CaCl}_2$ .	0.5 c.c. right flank.	All the mice died within 36 hours of gas gangrene.
(C) Mice 13 to 18.	Whole meat broth culture of <i>B. Welchii</i> .	0.25 c.c. right flank.	All mice died within 36 hours of gas gangrene.

## THE CONTAMINATION OF OYSTERS.

By A. T. NANKIVELL, M.D. (LOND.), D.P.H. (CAMB.),

*Medical Officer of Health, Poole,*

AND SERGT J. M. STANLEY, A.A.M.C.

(With 1 chart.)

### TOPOGRAPHY AND TIDES.

THE Harbour of Poole lies a few miles to the West of Bournemouth. It has an area approximately of 10,000 acres, but there is only a very narrow passage between its waters and the open sea. The width of this bottle-neck entrance is about 250 yards. The last published Admiralty chart gives the rate of the incoming tide through this narrow entrance at  $3\frac{1}{2}$  knots, and of the outgoing tide at 4 knots. The local opinion is that these official rates err on the moderate side. The rapid ebb and flow of the tides has an influence in the bacteriology of the oysters within the harbour.

The presence of the Isle of Wight to the eastward causes Poole Harbour and Bournemouth Bay to have four tides instead of the usual two. About three-quarters of an hour after the first high water the tide begins to ebb; but about  $2\frac{3}{4}$  hours later it is high again. The first flood tide begins  $4\frac{1}{2}$  hours before high water.

### OYSTERS.

For many years past the oysters dredged from Poole Harbour and from Bournemouth Bay have been contaminated. The reports of various Medical Officers have associated these oysters with cases of Enteric Fever. One of the present writers has known of Enteric Fever which occurred after eating Poole or Bournemouth oysters: no other possible cause of the disease could be ascertained in these cases. Samples of oysters have been taken infrequently and at irregular intervals in the past and examined bacteriologically: they were found by various bacteriologists to be polluted, more or less heavily, with *B. coli*, and sometimes with *Streptococci* and *B. welchii* (Table I). At the present time Poole oysters cannot be dredged except for relaying, and this ban upon them is of considerable financial loss to the district.

### THE PRESENT ENQUIRY.

Early in 1914 one of us (A.T.N.) was appointed to the post of Medical Officer of Health to the Borough and Port of Poole, and before the outbreak of war he had begun some research work on the subject of the pollution of

the harbour water and of the oysters, hoping by this to settle once and for all, by a thorough investigation, many details concerning the oyster pollution, which had been unsatisfactorily dealt with in the past by other observers in a partial manner and unconvincingly. This earlier research was necessarily interrupted, but on his return to the Borough the investigations were continued. The enquiry was directed towards:

1. The source of pollution of the oysters.
2. The nature of the infecting micro-organisms.
3. The discovery, if possible, of a suitable relaying place; and
4. In the absence of this, the effect of sterilisation by chlorinated sea-water.

Towards the cost of this research the Borough Council of Poole made a contribution of five pounds, and the Southern Sea Fisheries Board a grant of a similar amount. All the work referred to in this paper was done at the Poole Borough Laboratory; and we should like here to express our appreciation of the help given to us by our Laboratory Attendant, S. Marshall, who prepared most of our culture media, and aided us generally in the work.

#### TECHNIQUE.

Samples both of sea-water and of minced oysters were plated in neutral-red-bile-salts-peptone-lactose-agar. Both single strength and double strength were used—the latter for the plates of 10 c.c. and upwards. Of the sea-water we plated varying quantities from 0.1 c.c. to 15.0 c.c.; and examined in MacConkey Lactose tubes quantities from 20 c.c. to 100 c.c. We should like here to insist that plate cultivation gives more accurate results than can be obtained by the use of liquid media. If a 20 c.c. tube shows acid and gas it cannot accurately be estimated exactly how many lactose fermenters were present: if, however, four plates each of 5 c.c. are made, then the number of lactose fermenters can be seen and correctly counted. Gradually, as the work progressed, we used more plates and fewer tubes. Apart from this, our technique was that usually employed in the examination of samples of drinking water, as far as the sea-water was concerned. When dealing with the oysters we cleansed the outsides of the shells by thorough scrubbing under running water. The oysters were then opened with a sterilised knife, and the contents finely minced before removal from the concave shell. Each oyster was then emulsified in 100 c.c. of sterile saline. In dealing with oysters which were presumably polluted, plates of 0.5 c.c., 1 c.c. and 5 c.c. of the emulsion were made. When examining oysters after relaying we used six plates each of 5 c.c. and two plates each of 10 c.c. Saline emulsion corresponding to half an oyster was therefore plated and examined. On several occasions we checked our plate results by inoculating a series of MacConkey Lactose tubes.

## THE SOURCE OF POLLUTION OF THE OYSTERS.

The first oysters examined, taken from a bank just inside the harbour mouth, showed the presence of many lactose fermenters—about 700 per oyster, quite enough in our opinion to condemn them. (See Table I.) We had then to decide from what quarter this pollution came; did it originate within the harbour of Poole, or was it brought in from the outside?

Table I.

*Poole and Bournemouth Bay Oysters, 1914–1919.*

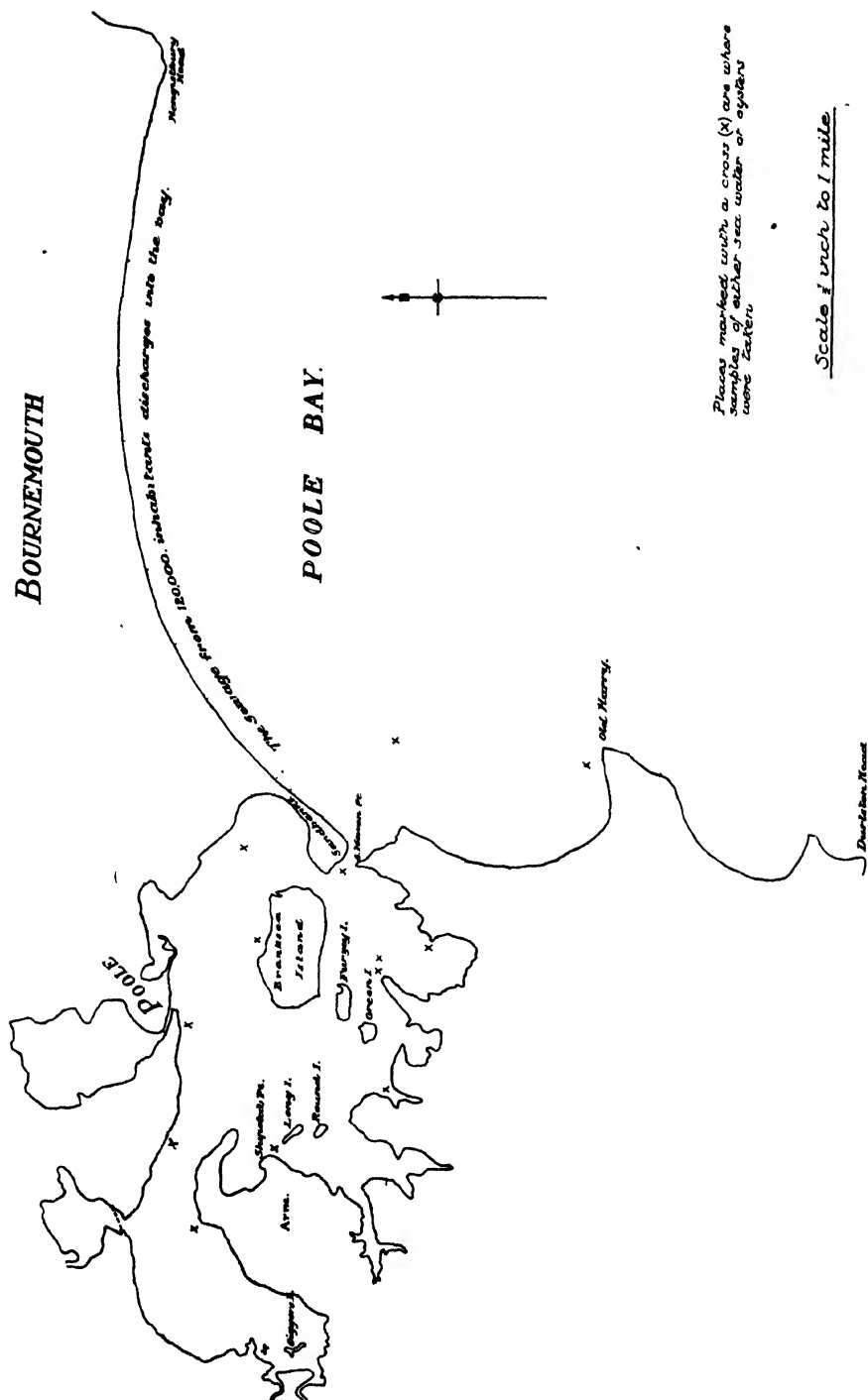
Date	Place	Bacteriological findings
May, 1914	Poole Harbour	700 lactose fermenters per oyster
June, 1914	Poole Harbour	<i>Streptococci</i> and <i>B. welchii</i> in $\frac{1}{10}$ of an oyster
June, 1914	Poole Harbour	1500 lactose fermenters per oyster
May, 1915	Poole Harbour	3000 lactose fermenters per oyster
Nov. 1915	Poole Harbour	<i>B. coli</i> and <i>streptococci</i> in $\frac{1}{2}$ c.c.
Sept. 1919	Poole Harbour	2400 lactose fermenters per oyster
Oct. 1919	Bournemouth Bay	1500 lactose fermenters per oyster

To settle this question we took half hourly samples of the water at the bottle-neck entrance to the harbour from Sandbanks Pier (see chart). The samples were taken throughout several tides. They were obtained in sterilised bottles from a depth of three feet below the surface by means of a specially designed plunger. The samples were sent by bicycle or motor-car to the laboratory four miles away, and were plated and tubed within a few hours of being taken. The first results were a surprise to us, and the experiment was therefore repeated over two other tides. The second series of plates however showed results which were generally constant—sufficiently so, at any rate, to prove without any doubt that the first incoming tide brought many lactose fermenters into the harbour; that on the second tide not so many were brought in, and that the water at outgoing tides was comparatively clean and free from micro-organisms (Table II). The pollution of the oyster beds therefore came in from the open sea; and not from the Town of Poole or from the rivers that flow into the top of the Harbour.

A knowledge of the local sewage outfalls and of the tides in the bay will explain how these faecal organisms can make their way in from the open sea. All the sewers of Bournemouth and of Poole discharge into the sea, and not into the harbour: the sewage is carried out in the ebb tide in the direction of the headland known as Old Harry. On arrival in this neighbourhood it is met by the strong incoming tide and pushed into Poole harbour. Subsidence of micro-organisms and certainly of macroscopical pieces of sewage takes place here, and this explains the finding that the water leaving the harbour is cleaner than that which comes in.

Having satisfied ourselves by these water samples, taken at Sandbanks, that the pollution came in on the first high tide, we next proceeded to ascertain how the water in different parts of the harbour was affected. We followed up

### Contamination of Oysters



the Main Channel taking samples at Salterns, at Hamworthy and off Russell Quay during different stages of the tide: these were infected, but, speaking generally, not so heavily as the water at Sandbanks. We found the same in the South Channel at Redhorn and at Goathorn, and in the Wych Channel opposite the Ower (Table II).

At these places we did not repeat our observations over several days, nor take so many samples—there seemed to be no need since none of the results were at variance with our earlier observations at Sandbanks.

#### IDENTITY OF MICRO-ORGANISMS.

We were not able to make a prolonged and thorough investigation of the lactose fermenting organisms which we isolated from the sea-water and from oysters. One of us (J.M.S.) had only four months to spend on non-military employment in this country before his return to Australia, and it was possible therefore systematically to examine only a small proportion of the organisms

Table II.

*Showing number of lactose fermenting organisms in 10 c.c. of the sea-water at various places at different stages of the tide.*

State of tide	Hours after low water	Sand-banks Pier	Sand-banks Pier	Sand-banks Pier	Salterns	Ham-worthy	Russell Quay	South Channel	Red-horn	Goat-horn	Wych	Ower	Ship-stall
Low water	0	0	1	0	—	—	—	—	—	—	—	—	—
	$\frac{1}{2}$	0	2	1	—	—	0	—	—	—	—	—	0
	1	0	3	0	—	—	0	—	—	—	—	0	0
Incoming tide	$1\frac{1}{2}$	1	2	1	—	—	1	—	1	—	1	0	0
	2	14	0	12	10	—	0	—	1	—	3	0	0
	$2\frac{1}{2}$	38	6	20	12	10	20	—	0	—	10	0	0
	3	56	14	24	12	30	44	—	26	—	12	0	0
	$3\frac{1}{2}$	86	20	32	14	16	8	—	14	—	—	0	0
	4	100	48	48	26	—	70	—	—	—	—	0	0
	$4\frac{1}{2}$	82	12	8	2	—	15	2	—	—	—	—	0
1st high water	5	6	1	2	—	—	7	1	—	—	—	—	0
	$5\frac{1}{2}$	2	0	1	—	—	0	28	—	1	—	—	0
	6	0	0	0	—	—	0	10	—	1	—	—	0
	$6\frac{1}{2}$	0	0	0	—	—	0	5	1	4	—	—	0
	7	0	0	0	—	—	0	—	0	1	—	—	0
	$7\frac{1}{2}$	0	0	0	—	—	0	—	0	—	—	—	0
	8	0	0	0	—	—	1	—	1	—	—	0	0
2nd high water	$8\frac{1}{2}$	1	1	0	—	—	0	—	—	—	—	0	0
	9	1	0	1	—	—	3	—	—	—	—	1	0
	$9\frac{1}{2}$	0	1	0	—	—	1	—	—	—	—	0	0
Outgoing tide	10	2	2	1	—	—	0	—	—	—	—	0	0
	$10\frac{1}{2}$	3	0	0	—	—	1	—	—	—	—	0	0
	11	1	1	0	—	—	0	—	—	—	—	—	0
	$11\frac{1}{2}$	0	0	0	—	—	0	—	—	—	—	—	0
Low water	12	0	0	0	—	—	—	—	—	—	—	—	0

*Note.*—Although no lactose fermenters were found in any quantity of 10 c.c. of Shipstall water examined, yet acid and gas were produced in 100 c.c. tubes. No change was however found in 75 c.c. tubes.

that we found during our routine examination of the plates and tubes. In all, 27 lactose fermenting organisms were subcultured. Their morphology was considered and they were examined regarding their behaviour to Gram's stain. They were tested also for motility. Their fermentation reactions were examined in peptone water containing lactose, saccharose, glucose and dulcite. Their Indole Reactions and their Vosges-Proskauer Reactions were deterred. It will be seen from Table III that the majority of these organisms proved to be "coliform" in nature.

Table III.

*Lactose fermenting organisms in sea-water.*

	Morphology	Glucose	Lactose	Saccharose	Dulcite	Indole	Litmus milk	Motility
1.	Gram-bacillus	A & G	A & G	A & G	A & G	+	A & C	+
2.	"	A & G	A & G	-	A & G	+	A & C	-
3.	"	A & G	A & G	-	-	-	A	?
4.	"	A & G	A & G	A & G	A & G	+	A & C	-
5.	"	A & G	A & G	A & G	A & G	+	A & C	+
6.	Gram + coccus	-	A & G	-	-	-	A	-
7.	Gram-bacillus	A & G	A & G	A & G	-	+	A & C	-
8.	"	A & G	A & G	A & G	A & G	+	A & C	-
9.	"	A & G	A & G	A & G	A & G	-	A & C	+
10.	"	A & G	A & G	A & G	-	+	A & C	+
11.	"	A & G	A & G	A & G	A & G	+	A & C	+
12.	"	A & G	A & G	-	A & G	+	A	-
13.	"	A & G	A & G	0	A & G	+	0	+
14.	"	A & G	A & G	A & G	A & G	+	0	-
15.	"	A & G	A & G	-	A & G	+	0	-
16.	"	A & G	A & G	0	A & G	-	0	?
17.	"	A & G	A & G	A & G	A & G	-	0	+
18.	"	A & G	A & G	-	A & G	+	0	-
19.	Gram-bacillus	A & G	A & G	0	A	-	0	-
20.	Gram + coccus	A	A	0	A	0	0	-
21.	Gram-bacillus	A	A	-	A	-	0	-
22.	"	A & G	A & G	A & G	A & G	-	0	+
23.	"	A & G	A & G	-	A	-	0	+
24.	"	A	A & G	0	A & G	+	0	-
25.	"	A	A	-	A	-	0	+
26.	Gram + coccus	A	A & G	-	A	-	0	-
27.	Gram-bacillus	A & G	A & G	-	A & G	-	0	-

*Note.*—A & G=acid and gas. +=Indole production. -=no change. 0=not examined. A=acid only. A & C=acid and clot. Nos. 1-12 are organisms obtained from sea-water in 1914. Nos. 13-27 are organisms recovered from sea-water and oysters in 1919. None of the organisms except No. 3 gave a positive Vosges-Proskauer Reaction.

It has been shown by other workers that intestinal organisms die rapidly in sea-water, and that a period of four or five days is sufficient even for a heavily infected sea-water to become practically sterilised.

The presence, therefore, of these lactose fermenters of the faecal or "coli-form" type showed that the pollution was recent. Not much interest attaches to the non-lactose fermenters which we isolated and subcultured. As is usual in enquiries of this nature, no *B. typhosus* or para-typhoid organisms were isolated.

## A SUITABLE RELAYING PLACE.

It seemed to us hardly likely that we should find a place anywhere within the harbour where the water was comparatively clean and suitable for the relaying and cleansing of oysters. If such a place was to be found it must be somewhere as far as possible from the harbour entrance; some place where the tides were not very swift, so that sedimentation and purification might have had time to take place before the water arrived at this hypothetical relaying ground. We thought that Redhorn or the top of the Wareham Channel might possibly be fairly clean, but they proved to be otherwise. Samples taken off Ower Farm and off Arne near Shipstall Point were however clean or nearly so—*B. coli* being present in 100 c.c. tubes but not in 75 c.c. tubes and five 10 c.c. plates being free from lactose fermenters (Table II). Owing to the difficulties of navigation, Ower is not a very accessible place, so we decided to relay some oysters off Shipstall Point near Arne. This place is situated at the top of the Wych Channel about three miles from the open sea. Between Arne and the sea lies Branksea Island and the water coming up to Arne takes a winding course around this: in addition, between Arne and the sea there are many acres of mud flats covered by rank weed and grass, which are awash with water when the tide is high: these practically form a filter for the sea-water. They slow the rate of the tide and must necessarily act as a mechanical filter to some of the water passing from the sea towards Arne.

Again, as this spot is so far from the open sea it is improbable that the water which forms any one high tide was actually in the open sea during the low water preceding that high tide—in other words if a single gallon of sewage contaminated water from the neighbourhood of Old Harry could be followed into Poole Harbour it would take several tides to find its way up to Shipstall Point. Indeed the actual movement of the water in these upper reaches is only slight, and this has been demonstrated by tide-floats. Something almost comparable to “storage” takes place in these places that are more remote from the sea, and self purification of the water is the natural outcome of this.

These natural conditions no doubt explain the fact that off Shipstall Point near Arne the sea-water was reasonably free from lactose fermenting organisms, and gave us a hope that the place might be suitable for the relaying of oysters. Twelve oysters were therefore dredged from the main channel. Six of them were brought straightway to the Laboratory and the remaining six were relaid in a buoyed net off Arne. The first half dozen were polluted, but not heavily; the half dozen which were relaid at Arne for seven days were clean with an average of only two lactose fermenting organisms per oyster. In view of this we decided not to pursue at present the intended part of our research which dealt with sterilisation by means of chlorine; but rather to accumulate as many facts as possible regarding the relaying at Arne.

The experiment was therefore repeated on several occasions, and always



with the same results—namely that the oysters before relaying were dirty and after relaying were clean—sometimes almost sterile (Table IV).

The greatest care was taken in the minute examination of these relaid oysters and a large number of plates of varying quantities were made. On one occasion for instance, 16 plates each of 2 c.c. were made from one oyster—practically one-third of the oyster was plated in small quantities—and only 13 lactose fermenters were found, corresponding to a total of 40 per oyster. On another occasion 0.9 part of an oyster was plated, giving a count of 12 lactose fermenters per oyster. On another occasion 25 c.c. of the emulsion were tubed in quantities of 1 c.c. in each of 25 tubes: acid and gas and a coliform organism were present in one tube—a total of four per oyster. In all 42 oysters after relaying were examined. The highest count observed was

Table IV.

*Results of relaying oysters at Shipstall.*

Before relaying		After relaying	
Date	Lactose fermenters per oyster	Date	Lactose fermenters per oyster
22. 9. 19	100	30. 9. 19	2
8. 10. 19	1600	18. 10. 19	14
8. 10. 19	1200	18. 10. 19	10
23. 10. 19	400	30. 10. 19	40*
—	—	4. 11. 19	10 (a)
4. 11. 19	400	12. 11. 19	15 (b)
4. 11. 19	700	12. 11. 19	40
12. 11. 19	2580	20. 11. 19	17 (c)
12. 11. 19	1050	20. 11. 19	12 (c)
22. 11. 19	590	2. 12. 19	3 (d)

(a) These oysters were found off Arne. They had not been laid there by us.

(b) 5 oysters mixed. Quantities equal to one-third of an oyster plated.

(c) 5 oysters mixed. Quantities equal to nine-tenths of an oyster plated.

(d) 5 oysters mixed. Quantities equal to one-half of an oyster plated.

40 *B. coli* per oyster: the lowest was 2 per oyster. The average was 16 per oyster. As the result of these findings we have recommended that Shipstall Point off Arne is a safe place for the relaying of oysters; and have been able to give an assurance that oysters which have been relaid there for a week will be of reasonable bacteriological purity.

## CONCLUSION.

1. The contamination of the water in Poole Harbour comes in with the flood tide from the open sea, and does not originate within the Harbour.

2. The nearer the sea and the more rapid the current, the greater is the pollution of the Harbour water.

3. The large oyster beds near the harbour entrance and in the main channel are polluted, and the oysters in them contain many organisms derived from sewage.

4. These oysters can be cleansed by relaying off Shipstall Point near Arne where the water is comparatively free from sewage organisms.

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